Characterisation of the B-lymphocyte response in delayed-type piperacillin hypersensitivity reactions.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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Declaration

I declare that the work presented in this thesis is all my own work and has not been submitted for any other degree.

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To

Fatimah and Aayan.

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ABBREVIATIONS

A260	Absorbance at 260nm
A280	Absorbance at 280nm
ACN	Acetonitrile
ADR	Adverse drug reaction
ALP	Alkaline phosphatase
APC	Antigen presenting cells
APC	Allophycocyanin
BSA	Bovine serum albumin
Breg	B regulatory cells.
CCR	Chemokine receptor (C-C) motif
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
Cpg-dna	Cytosine phosphate guanosine deoxyribonucleic acid
cpm	Counts per minute
CSA	Cyclosporin
CTL	Cytotoxic T lymphocyte
CYP	Cytochrome P450 enzyme
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DHR	Drug hypersensitivity reaction
DILI	Drug-induced liver injury
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNCB	Dinitrochlorobenzene
DNFB	Dinitrofluorobenzene
DRESS	Drug reaction with eosinophilia and systemic symptoms
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminiscence
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot

FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum VII
FITC	Fluorescein isothiocyanate
HBSS	Hanks balanced salt solution
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
HRP	Horse radish peroxidase
HSA	Human serum albumin
IFN-γ	Interferon-gamma
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LPS	Lipopolysaccharide
LTT	Lymphocyte transformation test
MALDI-TOF	Matrix assisted laser desorption ionisation-Time of flight.
MAMP	Microbe associated molecular patterns
MHC	Major Histocompatibility complex
Mins	Minutes
MRM	Multiple reaction monitoring
NHS	National health service
NK	Natural killer
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
pH	Power of hydrogen VIII
pi	Pharmacological interaction
pKa	Acid dissociation constant
Poly (I:C)	Polyinosinic acid:polycytidylic

RPMI	Roswell Park Memorial Institute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFC	Spot forming cell
SI	Stimulation index
SIT	Specific immunotherapy
SJS	Stevens-Johnson syndrome
TCR	T-cell receptor
TEN	Toxic epidermal necrolysis
Th1	Type 1 helper cell
Th2	Type 2 helper cell
TNF-α	Tumour necrosis factor-α
Tregs	Regulatory T cells
TT	Tetanus toxoid
UK	United Kingdom
w/v	weight/volume
WHO	World Health Organisation

ABSTRACT

Adverse drug reactions remain a major health issue with delayed type hypersensitivity reactions developing in a high number of individuals. The cellular immunological processes that underlie drug-specific responses in hypersensitive patients have been previously described; however the involvement of the humoral immune system has not been studied in great detail. Consequently, this thesis explores the nature of the piperacillin-specific B cell response in hypersensitive patients and compares the cellular and humoral immune response that develops in patients with cystic fibrosis (CF) exposed to repeated courses of the drug.

Initial studies involved characterization of B cell proliferation, B cell phenotype and the nature of total and drug-specific IgG antibody secretions using peripheral blood mononuclear cells (PBMC) from hypersensitive patients. For comparison PBMCs from 2 groups of individuals were assessed: piperacillin naïve healthy volunteers and piperacillin tolerant patients with CF. ELISA, and ELISpot were used to detect piperacillin-specific B cells responses and IgG secretion. T lymphocyte proliferation was assessed with the lymphocyte transformation test (LTT). T lymphocytes from hypersensitive patients, but not tolerant patients or naïve donors were stimulated to proliferate in the presence of the drug. The peak concentration for T cell activation was 1 mM. Phenotypic assessment of hypersensitive patients B-cells revealed an increase in CD19+CD27+ expression in response to piperacillin treatment *in vitro*. IgG secreting immortalized B-cell lines also expressed a pure $CD19^{+}CD27^{+}$ phenotype. Piperacillin stimulation of hypersensitive patient PBMC also led to an increase in the secretion of IgG. In contrast, IgG secretion was not detectable following piperacillin stimulation of PBMC from tolerant patients and healthy controls.

Western blotting and mass spectrometric methods were applied to characterize β-lactam-protein covalent binding. Bovine serum albumin (BSA) binding was time- and concentration-dependent with hapten densities (i.e., the extent of selective lysine residue modification) and anti-piperacillin antibody binding affinity increasing with increasing molar ratios. Lysine residues in BSA at positions 4, 12, 131, 132, 136, 211, 431, 524, and 537 were modified by piperacillin. Epitope profiles also showed similar lysine residues were modified with amoxicillin, benzylpenicillin and flucloxacillin though the extent of ionisation at each site of modification was drug-dependent. A hapten inhibition ELISA used to assess the specificity of the antidrug antibodies revealed the total antibody binding to aztreonam, amoxycillin, benzylpenicillin and penicillin V BSA adducts. This indicates a lack of cross-reactivity with piperacillin-specific IgG antibodies.

Subsequently, LTT and ELISA were employed to screen the piperacillin-specific T cell response and IgG antibodies during piperacillin therapy. It was established that piperacillin-specific T cells were detectable on and following clinical diagnosis of hypersensitivity. Moreover, piperacillin-specific T cell responses were detected in a small number of patients currently classified as drug tolerant. A significant difference in piperacillin-specific IgG was observed when plasma form LTT positive and negative blood samples were compared. LTT positivity was associated with higher levels of piperacillin-specific IgG. Furthermore, a significant decrease in piperacillin-specific IgG was seen 24 h post-desensitisation (graded drug challenge).

Piperacillin-specific T cell clones isolated from hypersensitive patients were used to explore the effect of plasma bearing anti-piperacillin IgG on the T cell response. Eleven piperacillin-specific CD4+ and CD8+ T-cell clones were generated from 2 hypersensitive patients. All clones were stimulated to proliferate with piperacillin in a concentration-dependent manner. IFN-γ and IL-5 secretion was seen to predominate following piperacillin stimulation. There were no differences in piperacillin-specific T-cell proliferation when piperacillin-specific antibody bearing plasma and plasma from naive volunteers were compared. However, attenuation in IFN-γ secretion was observed with plasma bearing anti-piperacillin antibodies alone.

Collectively, the data presented in this thesis begins to describe the different components of the drug-specific humoral and cellular immune response that develops in piperacillin hypersensitive patients with CF.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Table of contents

Figures

Tables

1.0 Introduction

The growth in the repertoire of pharmaceutical agents used in the treatment of disease has ensured that adverse drug reactions (ADRs) remain a major health issue, with recent studies in the UK indicating that ADRs are a common clinical problem accounting for 2 - 6% of all hospital admissions (Pirmohamed, James et al. 2004). ADRs have been found to affect the skin, liver, blood, kidney and lungs (Mockenhaupt and Schopf 1996, Kudo 2007, Uetrecht and Naisbitt 2013) and have been shown to contribute significantly to an increase in healthcare costs due to an extended length of hospital stay and the requirement of additional clinical investigations in more serious cases (Suh, Woodall et al. 2000, Pirmohamed, James et al. 2004, Sultana, Cutroneo et al. 2013). In other cases the outright withdrawal of the offending agents from distribution and clinical use such as the selective serotonin reuptake inhibitor (SSRI) Zelmid withdrawn from clinical use in 1983 due to adverse neurological effects (Mulinari 2015), and the appetite suppressant FEN-PHEN (Fenfluramine ("fen") and phentermine ("phen")) withdrawn due to adverse cardiovascular effects in 1997 by regulatory agencies (Connolly, Crary et al. 1997, FDA 1997) led to huge financial losses for the pharmaceutical companies involved (Schindler 2011).

The clinical impact of ADRs has thus necessitated the development of ADR monitoring and reporting systems collectively referred to as pharmacovigilance. Pharmacovigilance as defined by the world health organization is 'The science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other drug related problem' (WHO). ADRs are a complex disease requiring a multidisciplinary approach to understand their pathomechanisms. Basic and clinical pharmacology,

chemistry, immunology, clinical medicine, toxicology, epidemiology, and pharmacogenetics have all contributed to our understanding of the disease pathogenesis (Biffignandi 2009).

The objective in this chapter is to review current pharmacological, immunological and chemistry literature relevant to the study of drug-induced hypersensitivity reactions.

1.1 Adverse drug reactions: Definitions

An adverse drug reaction (ADR) may be defined according to the WHO as "a response to a drug that is noxious and unintended and occurs at doses normally used in man for prophylaxis, diagnosis or therapy of disease, or for modification of physiological function (WHO, 1972). Edwards and Aronson in their definition of an adverse reaction tried to take into account the fact that while some unpleasant reactions may be mild in nature, errors in medication, contaminants and supposedly inactive excipients may be responsible for the adverse effects being observed. They thus defined ADRs as "an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product." (Edwards and Aronson 2000)

1.1.1 Epidemiology and risk factors

Adverse reactions to drugs occur frequently, and in many cases are difficult to avoid. Data has shown that a sizeable proportion of hospitalized patients experience a form of ADR, which also constitutes one of the most important reasons for hospital admission (2-6%) with a substantial increase in the elderly. Drug-related hospital admissions in

individuals aged over 75 accounts for approximately 30% of all hospitalized patients. (Runciman, Roughead et al. 2003, Gamboa 2009). Due to the resultant significant morbidity, mortality and socioeconomic impact of ADRs, several studies have been carried out to quantify the impact of ADRs. The incidence of ADRs in hospitalized patients has been estimated to range from 1.8% in a study conducted at the LDS Hospital in Salt Lake City, which identified 731 reactions among 36,653 hospitalized patients during an eighteen month period, 6.5% in the United Kingdom, to a maximum of 15.1% in a meta-analysis of thirty three studies conducted in the USA over a thirty year period, from 1966-1996 (Classen, Pestotnik et al. 1991, Lazarou, Pomeranz et al. 1998, Pirmohamed, James et al. 2004). Epidemiologic data regarding the prevalence of drug allergies suggested a varied prevalence among different populations, which could be attributed primarily to the different criteria for inclusion and methodologies employed (Gomes and Demoly 2005). The apparent lack of reliable data generally obtained from respondents by self-reporting from the general population prevents the direct comparison of the prevalence between hospitalized and ambulatory patients (Gomes, Cardoso et al. 2004, Ensina, Amigo et al. 2010).

Several classes of drugs including the beta lactams, the non-steroidal anti-inflammatory agents, and anti-seizure drugs are associated with a high incidence of ADRs. β-lactam antibiotics have been recognized as the most frequent cause of immunological reactions (Rodriguez-Pena, Antunez et al. 2006).

Various factors may play a role in the development of adverse drug reactions, with a majority typically due to an extension of the expected pharmacological activities of these pharmaceuticals. While this may be the case in a large proportion of reactions, inter-patient pharmacokinetic and pharmacodynamic variability, pharmacological,

immunological and genetic factors are also involved in the development of ADRs. These factors could be related to the patient age or sex, the drug employed, and environmental or social related issues, as well as disease related factors (Alomar 2014) and genetic predisposition (Pirmohamed 2006, Park, Kim et al. 2014).

Patients at the extremes of age have been shown to be predisposed to the development of certain forms of ADRs. Elderly patients that have multiple disease states are exposed to multiple drugs and thus are particularly susceptible to the development of ADRs. Infants and very young children are also at risk due to their low level of organ and tissue development such as immature renal tubular function, physiologic hypoalbuminemia, low body fat, and immature blood brain barrier in neonates below the age of 8 weeks (Anderson and Lynn 2009, De Gregori, De Gregori et al. 2009, Ibanez, Lopez-Bermejo et al. 2009, Schoderboeck, Adzemovic et al. 2009). This leads to an inability to readily metabolise a host of drugs. Like in infants, the elderly patients the mechanism of drug absorption, distribution, metabolism, and elimination is less likely to be studied widely (Kaushal, Bates et al. 2001, Gamboa 2009).

Gender differences play a role in the action of many drugs and thus affect the way the body deals with the drugs, altering drug pharmacokinetics and pharmacodynamics in the process. Sex differences have been implicated in the severity and frequency of ADRs to antiretroviral agents (Ofotokun and Pomeroy 2003). Moreover hepatic enzyme activity, specifically CYP3A4 leading to the enhanced metabolism of midazolam in Chinese women when compared to men (Labbe, Sirois et al. 2000, El-Eraky and Thomas 2003), and in the effect of colchicine which may selectively affect fertility in males but not in females (Sternberg and Hubley 2004) are poignant examples. Pregnancy, menopause and menstruation which are issues confined specifically to females could also have profound

drug effects (Mitchell, Smith et al. 2009).

Alcohol consumption and smoking affects the metabolism of many drugs, and in turn are capable of altering susceptibility to ADRs. Chronic alcohol consumption activates enzymes that transform drugs and are able to alter the predicted actions of these agents hence producing effects capable of causing damage to the liver and other organs (Alomar 2014) . Smoking also affects liver enzyme activity as it is a potent inducer of the hepatic cytochrome P-450 (CYP) isoenzymes 1A1, 1A2, and, possibly, 2E1 (Tomlinson, McMahon et al. 2005).

There is evidence to suggest that ethnicity controlled by genetic factors exerts a substantial influence on drug response and action. In the evaluation of risk factors for ADRs in patients on angiotensin converting enzyme (ACE) inhibitors, ACE related angioedema was found to be more prevalent in African Americans when compared with other ethnic groups (Morimoto, Gandhi et al. 2004). In another study the risk of cough with blood pressure lowering drugs was three times greater in black patients than non-black patients (Grouzmann, Livio et al. 2009). Recently, the human leukocyte antigen (HLA) genes have been identified as genomic markers of predisposition to several forms of ADRs; for example abacavir hypersensitivity is associated with HLA-B*57:01 (Mallal, Phillips et al. 2008). Further associations include allopurinol and HLA-B*58:01 in Hong Kong Han Chinese and Portuguese (Somkrua, Eickman et al. 2011, Chiu, Hu et al. 2012, Goncalo, Coutinho et al. 2013), carbamazepine and HLA-B*15:02 (Chung, Hung et al. 2004), or HLA-A*31:01 in the Han Chinese and European populations respectively (McCormack, Alfirevic et al. 2011, Ozeki, Mushiroda et al. 2011, Yip, Marson et al. 2012).

1.1.2 Classification of ADRs

ADRs are usually classified into two types A and B based on their perceived dose dependence and predictability. Further classifications have been proposed to include a combination of dose and time related reactions associated with cumulative dose, time-related reactions alone, withdrawal-induced reactions and reactions attributable to the failure of drug therapy usually due to inadequate dosing and drug interactions (Edwards and Aronson 2000, Aronson and Ferner 2003).

i. Type A ADRs: are due to exaggerations in the drugs known pharmacological effect and are usually dose dependent, predictable and account for a majority of ADRs. Type A reactions make up approximately 85% of all ADRS and include reactions like GIT bleeding after treatment with NSAIDs, and interactions with other drugs and/or any underlying illnesses. Generally type A ADRs are dose-dependent and are related to the pharmacokinetic properties of the drug. A reduction in the dose or withdrawal of the offending drug usually leads to a resolution of the ADR (Pirmohamed, Breckenridge et al. 1998).

ii. Type B ADRs: These reactions are usually idiosyncratic and unrelated to the drugs known pharmacology. They are less common and tend to be more serious than type A reactions, comprising about 10-15% of all ADR (Hausmann, Schnyder et al. 2010). Type B reactions were previously thought to be unrelated to the dose, and unpredictable as the mechanism was ill-defined. Many type B reactions provoke the host immune system as a part of the disease profile. The involvement of drug-specific immunoglobulins, T-lymphocytes and other components of the adaptive immune system have been identified as important mediators in the pathogenesis of the ADRs (Naisbitt, Farrell et al. 2003).

1.2 Drug hypersensitivity reactions

Drug hypersensitivity reactions make up about one sixth of all ADRs (Pichler, 2007). These reactions have been earlier classified pathophysiologically by Gell and Coombs into four types namely; Type I, Type II, Type III, and Type IV. This classification did not take into account the large body of drug reactions that have now been defined clinically such as pseudo-allergic reactions, primarily antibody-mediated reactions and T cell mediated reactions which serve as a basis for more recently proposed classifications. The most recent classification utilizes the basic structure proposed by Gell and Coombs, and also goes further to include those that were excluded earlier (Descotes and Choquet-Kastylevsky 2001, Posadas and Pichler 2007).

1.2.1 Type I hypersensitivity reactions

Type I hypersensitivity is also known as immediate or sometimes anaphylactic hypersensitivity. The reaction may involve skin (urticaria and eczema), eyes (conjunctivitis), nasopharynx (rhinorrhea, rhinitis), bronchopulmonary tissues (asthma) and the gastrointestinal tract (gastroenteritis). Responses usually occur within 30–60 min but can be extremely quick (within a few minutes) and may result in anaphylaxis (systemic type I hypersensitivity). A late onset reaction may occur in some cases, 3 or 4 hours after allergen exposure. Symptoms could range from minor itching and inflammation to death. The effector mechanism has as its primary component mast cells and basophils and the response could be modified by platelets, neutrophils or eosinophils, though a biopsy of the reaction site has revealed mainly mast cell and eosinophil involvement.

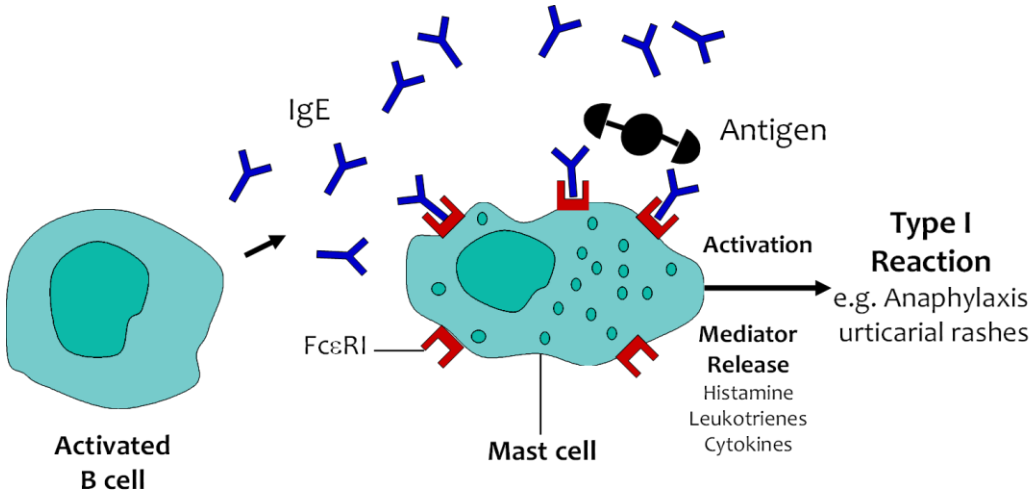


Figure 1.1. Type I hypersensitivity reactions. Diagram showing the pathway of Type I hypersensitivity reactions which involves the release of IgE from activated B cells, mast cell degranulation and the subsequent release of mediators of inflammation leading to type I reaction manifestations.

Type I reactions are mediated by IgE, which is preferentially produced by most cells in response to specific allergens. These antigen specific IgE are able to bind high affinity Fc-IgE receptors on mast cells that at are able to bind antigen or drug-specific IgE to a high degree. The binding of IgE antibodies to complementary FcεRI receptors on mast cells via their Cε3 region leaves both antibody combining sites free to interact with the complementary allergenic determinants. This leads to cross-linking of the cell bound antibodies, degranulation of the anchoring mast cells and and triggers the release of preformed and newly formed mediators of hypersensitivity such as proteoglycans, serine proteases, leukotrienes (Yamasaki & Saito, 2005) and a host of other factors.

Drugs implicated in the pathophysiology of type I allergic reactions include antibiotics (penicillins, cephalosporins, quinolones), neuromuscular blocking drugs, some nonsteroidal anti-inflammatory drugs such as pyrazolones, trimethoprim, sulphamethoxazole, proton pump inhibitors, heparin, insulin, *L*-asparaginase, etanercept,

and chimeric human–animal monoclonal antibodies used for therapy. Diagnosis of Type I hypersensitivity reactions is traditionally carried out using provocation testing which involves a topical challenge with certain antigens closely followed by observation of the response of the patients dermis to the challenge. This test is associated with some risk as some antigens result in severe anaphylaxis (Smith 1992). Alternative methods which could be employed to determine hypersensitivity include skin prick or patch test, radioallergosorbent tests (RASTs), leukocyte histamine release assays, surface markers for basophil activation, and leukotriene release tests (Williams, Dolen et al. 1992, Primeau and Adkinson 2001).

In the determination of the likelihood of a culprit drug to cause immediate hypersensitivity, the basophil activation test (BAT) and the fluorescence enzyme immunoassay were found to be less sensitive than the skin prick test, with the BAT being the least specific (Khan, Ueno-Yamanouchi et al. 2012). The RAST is also not as sensitive as the skin prick test and produces high variability between tests (Roberts-Thomson, McEvoy et al. 1993, Yunginger, Ahlstedt et al. 2000)

1.2.2 Type II hypersensitivity reactions

Type II hypersensitivities are also known as cytotoxic hypersensitivity and antibody-dependent cytotoxicity. They are rare reactions with serious and potentially life threatening implications. These reactions are mediated by IgG and IgM antibodies which bind to self-cell surface molecules forming complexes which activate the complement system, this triggers opsonisation, red blood cell agglutination, cell lysis and death (Warrington, Watson et al. 2011). Due to the involvement of multiple underlying mechanisms, a host of tissues and organs may be involved, and while endogenous

antigens are those generally involved in the induction of the reaction, drugs are also known to provoke their own forms of type II hypersensitivity which include immune haemolytic anaemia, thrombocytopenia and granulocytopenia. Reaction times can range from minutes to hours, but drug-induced immune thrombocytopenia generally manifests after 5 to 8 days of exposure to the culprit drug, or after a single exposure in patients previously exposed to the same drug (Pichler, Adam et al. 2010). Drugs which have been found to cause type II hypersensitivities include antibiotics (penicillins, vancomycin, and some cephalosporins), sulphonamides, quinidine, quinine, α-methyldopa, propylthiouracil, gold salts, and acetaminophen.

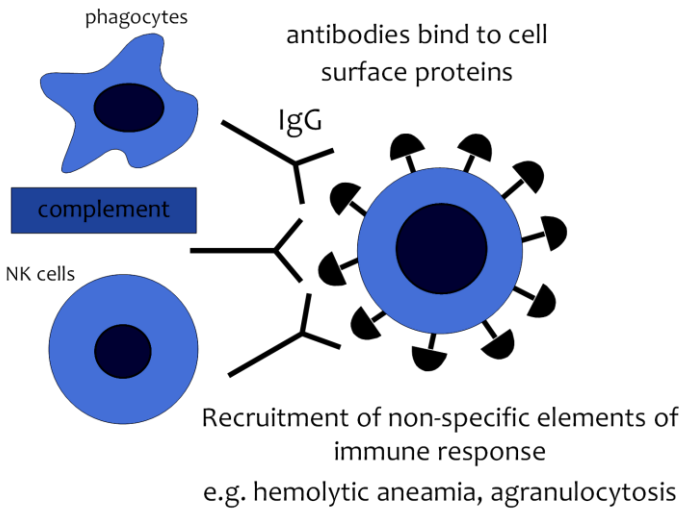


Figure 1.2. Type II hypersensitivity reactions. The binding of IgG and IgM antibodies to cell surface molecules forms complexes capable of activating the complement system. The end result being cell termination via phagocytes and NK cells.

1.2.3 Type III hypersensitivity reactions

They are also referred to immune complex deposition hypersensitivity. They are mediated by soluble immune complexes of antigen with antibodies generally of the IgG isotype. The similarity between type II and III hypersensitivity reactions lie in the dependence of both reactions on the formation of immune complexes, but their target

structures and physiological effects are distinct. A consequence of immune complex deposition in tissues is a reaction which may lead to mast cell degranulation, leukocyte chemotaxis, and inflammation induced by the cell influx.

After exposure, reactions can develop to antigens which can be endogenous (DNA/anti-DNA/complement) or exogenous (filarial worms, dengue virus, microbial antigens, drugs) in nature. Post exposure, reactions may develop over a period of-about 3–10 h against antigens. Drugs implicated include dapsone, (erythema nodosum leprosum in the skin of leprosy patients), penicillins (Jarisch–Herxheimer reaction in syphilitic patients), β-lactams which induce hypersensitivity vasculitis , cotrimoxazole, NSAIDs and some monoclonal antibodies (Alomar 2014).

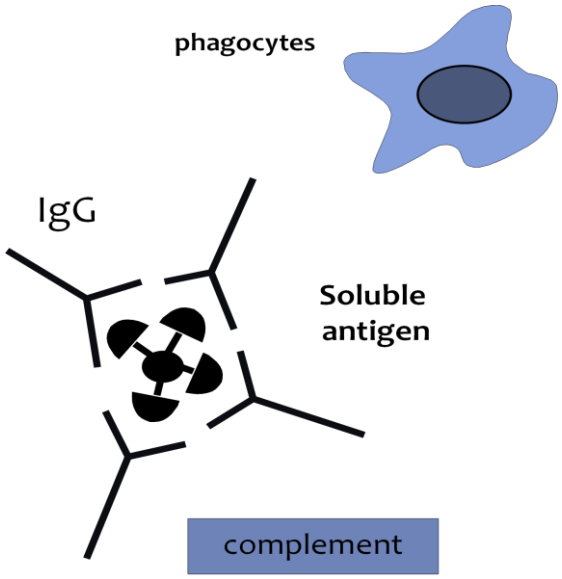


Figure 1.3. Type III hypersensitivity reactions. IgG antibody forms immune complexes and are targeted by phagocytes leading to either mast cell degranulation, leukocyte chemotaxis or inflammation.

There remains controversy as to the ultimate effector molecules in both Type II and III hypersensitivity reactions. Generally the roles of drug-specific effector T cells have not

been studied. It is theoretically possible that IgG antibodies play a bystander effector role and that T-cells are responsible for the tissue injury through the release of cytokines. Alternatively IgG antibodies might exert a regulatory function. The latter is discussed in detail later in this chapter.

1.2.4 Type IV hypersensitivity reactions

Also referred to as T-cell-mediated, delayed drug hypersensitivity reactions. Unlike the other types of hypersensitivity reaction, which involve antibodies, type IV reactions are mediated by antigen-specific T-cells. Type IV hypersensitivity refers to a number of clinical reactions, with the cellular immune base being their main common factor. These reactions are caused by the overstimulation of T cells and monocytes/macrophages leading to the release of cytokines that cause inflammation, cell death and tissue damage. They are the second most common type of hypersensitivity reaction. The cellular response generally develops 2 or more days after antigen exposure which led to the inclusion of the term 'delayed' and also distinguishes the response from immediate reactions, which develops considerably earlier, within minutes and peaking after a few hours. Four subdivisions based on effector cells and mediators involved and the resulting cutaneous manifestations have recently been proposed. These include; Type IVa which is mediated by macrophage activation characterized with Th1 involvement and high IFN-γ/TNFα secretion. Type IVb reactions are mediated by eosinophils and Th2 secreting T-cells with high IL-4/IL-5/IL-13 secretion. Type IVc reactions are mediated by CD4 and cytotoxic CD8 cells which are relied upon as effector cells, and appear to occur in all drug-related delayed hypersensitivity reactions. Finally type IVd reactions are mediated by T-

cells, which secrete IL-8 when activated by drug, that recruit neutrophils (Pichler, W.J 2007).

Representative cutaneous reactions range in severity from maculopapular rash to Stevens Johnsons syndrome and toxic epidermal necrolysis (SJS/TEN), although the response is generally heterogeneous with the tendency for reactions to overlap which blurs the clinical picture. Primary methods used to establish a Type IV hypersensitivity in susceptible individuals are via delayed skin testing (Ahmed and Blose 1983), the lymphocyte transformation test (Pichler and Tilch 2004, Earnshaw, Pecaric-Petkovic et al. 2014) and the enzyme-linked immunospot (ELISPOT) assay which is a technique that permits the analysis of the frequency of antigen-specific, cytokine producing cells (El-Ghaiesh, Monshi et al. 2012, Naisbitt, Nattrass et al. 2014).

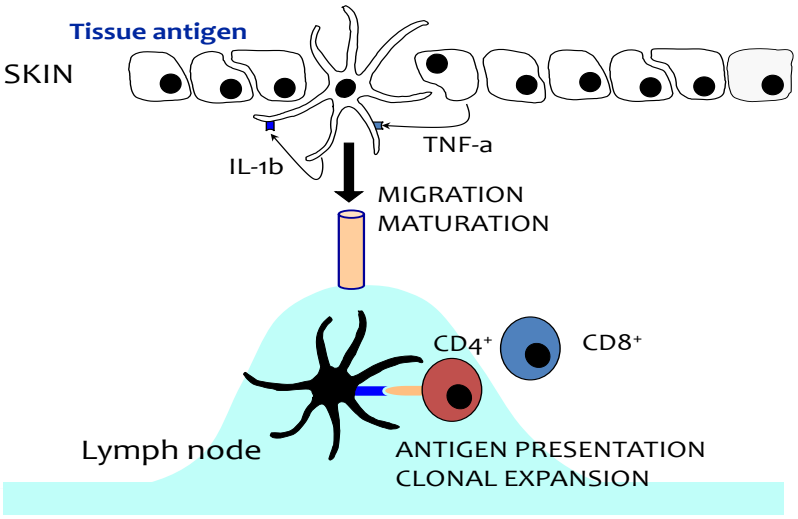
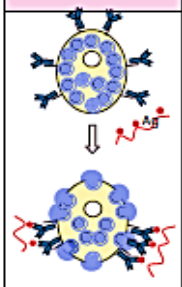
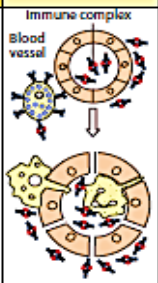
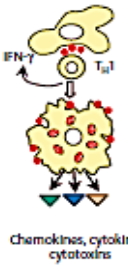
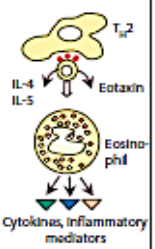
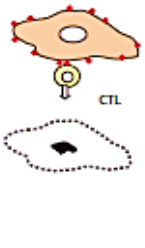
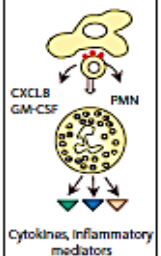


Figure 1.4. Type IV hypersensitivity reactions. These reaction are mediated via antigen specific T cells activation. CD4+ or CD8+ T-cells are activated, and monocytes and macrophages may be activated to release cytokines. The ensuing inflammation leads to tissue damage and cell death.

Drug hypersensitivity resulting in end organ effects has been demonstrated with the development of drug-induced kidney damage manifested as interstitial nephritis, lung

involvement and in murine models of drug-induced liver injury (Neilson 1989, Naisbitt, Farrell et al. 2005, Erhardt, Biburger et al. 2007, Pichler 2007, Erhardt and Tiegs 2010). These hypersensitivity reactions have shown mononuclear cell involvement with drug-specific activation of T cells featuring prominently in the immune regulatory and effector phases of the delayed hypersensitivity reactions. The end organ damage associated with these reactions differentiates them from current classifications which focus primarily on the cutaneous manifestations. Hence, with T cells playing a prominent role the recommendation is to define a new class as Type IVe specifically for these reaction types.

Table 1.1 Revised Gell and Coombs classification of hypersensitivity reactions. All reactions are T cell regulated but differ in their effector functions. Type I to III reactions being mediated by antibodies while Type Iva to IVd are mediated by more T cell/cytokine dependent functions (Adapted from; Drug hypersensitivity, Pichler WJ, 2007).

	Type I	Type II	Type III	Type IVa	Type IVb	Type IVc	Type IVd
Immune reactant	IgE	IgG	IgG	IIFN-γ, TNF-α (T_H1 cells)	IL-5, IL-4/IL-13 (T_H2 cells)	Perforin/ granzyme B (CTL)	CXCL8, GM-CSF (T cells)
Antigen	Soluble antigen	Cell- or matrix-associated antigen	Soluble antigen	Antigen presented by cells or direct T-cell stimulation	Antigen presented by cells or direct T-cell stimulation	Cell-associated antigen or direct T-cell stimulation	Soluble antigen presented by cells or direct T-cell stimulation
Effector	Mast cell activation	FcR+ cells (phagocytes, NK cells)	FcR+ cells Complement	Macrophage activation	Eosinophils	T cells	Neutrophils
							
Example of hypersensitivity reaction	Allergic rhinitis, asthma, systemic anaphylaxis	Hemolytic anemia, thrombocytopenia (e.g., penicillin)	Serum sickness, Arthus reaction	Tuberculin reaction, contact dermatitis (with IVc)	Chronic asthma, chronic allergic rhinitis Maculopapular exanthema with eosinophilia	Contact dermatitis Maculopapular and bullous exanthema Hepatitis	AGEP Behçet's disease

1.2.5 Clinical manifestations of cutaneous drug hypersensitivity

Although the clinical manifestations of ADRs are varied and involves many organs, the skin remains the major organ affected during the course of drug hypersensitivity reactions (Merk, Baron et al. 2007, Merk 2009). Cutaneous manifestations of ADRs include maculopapular exanthema, acute generalised exanthematous pustulosis, drug reactions with eosinophilia and systemic symptoms, Stevens Johnsons syndrome and toxic epidermal necrosis.

i. Maculopapular Exanthema (MPE): Maculopapular exanthema is a common cutaneous manifestation of drug hypersensitivity, and as a response to viruses and bacterial agents. In fact they have been estimated to account for approximately 95% of all drug-induced eruptions (Bigby 2001). Viruses such as cytomegalovirus (CMV), and several types of herpes (varicella zoster, HHV6 and the gamma herpes virus), bacterial diseases, lymphoma and graft versus host disease are also not excluded from the precipitation of exanthemas (Fernandez, Canto et al. 2009).

Skin presentations include erythematous macular or papular eruptions which often start on the trunk and subsequently spread to the extremities. Individuals also present with lesions which may also be found at the extremities. Studies have shown that the cell migration in drug-induced MPE generally favours drug-specific T cells which are capable of promoting the skin inflammatory reactions through the release of cytokines such as IL-5, TNF-α, IFN-γ and certain chemokines such as eotaxin (Yawalkar 2005). MPE symptoms which arise due to drug administration usually have an acute onset then resolve spontaneously, after a period which may range from hours, or up to 2 or 3 weeks after drug exposure. Continuation of drug administration after the onset of symptoms will lead to persistence and possible exacerbation of the reaction. These symptoms tend to

appear again after resolution and re-administration of the drug even after extended periods.

ii. Acute Generalized Exanthematous Pustulosis (AGEP): Is an acute skin eruption which is characterized by fever and the appearance of pustules that overlay edematous erythema. AGEP is induced in 90% of cases by drugs (Beltraminelli, Lerch et al. 2005) with an incidence of 3 to 5 cases per million per year and a mortality rate of 5%. There is a predominance of antibiotics as the causative agent in the development of AGEP, constituting over 80% of cases, though other drugs may be implicated such as hydroxychloroquine, cotrimoxazole, diltiazem, terbinafine and carbamazepine. Time of onset to resolution of the rash can be from as short as 24 hours. Resolution takes approximately 2 weeks. Activated drug-specific cytotoxic T cells are involved in tissue destruction (Schmid, Kuechler et al. 2002).

iii. Drug Reactions with Eosinophilia and Systemic Symptoms (DRESS). Also referred to as hypersensitivity syndrome or drug-induced hypersensitivity syndrome (DIHS) is a severe life- threatening adverse drug reaction. Clinical manifestations in skin reactions can be similar to MPE and AGEP but a visceral involvement with damage to internal organs and extended period of disease symptoms from the time of drug exposure (after 2-8) weeks are distinguishing features of DRESS. The incidence of DRESS ranges from 1:1000 to 1:10000 with a majority of patients presenting a prominent eosinophilia (Lopez-Rocha, Blancas et al. 2014). Viruses have also been implicated in the pathogenesis of DRESS with a rise in reactivation of herpes viruses, a rise in anti-HHV-6 IgG titers and the presence of HHV-6 DNA 2–3 weeks after the onset of rash noted in many patients (Baldo and Pham 2013).

$CD8^+$ T lymphocyte activation, enhanced secretion of TNF-α and IFN-γ and increased cutaneous homing have been identified as hallmarks of HHV-6 induced DRESS. DRESS inducers sulfamethoxazole, carbamazepine and allopurinol encourage viral proliferation and have been hypothesized as culprits that profile the kinetics of HHV-6 induced DRESS and the DRESS flare-ups which occur after switching from a culprit anticonvulsant to another structurally unrelated anticonvulsant (Picard, Janela et al. 2010, Joshua, N. et al. 2012).

iv. Stevens Johnsons Syndrome and Toxic epidermal necrolysis (SJS/TEN): These hypersensitivity conditions collectively referred to as (SJS-TEN) are both potentially fatal. Reactions are rare with incidences of usually less than two per million per year (Harr and French 2010) and involve damage to both the skin and mucus membrane. The extent of skin involvement remains the main distinguishing factor between the two. In SJS skin detachment is usually less than 10% of the total body surface while TEN patients manifest necrosis to at least 30% of their cutaneous tissue (Bastuji-Garin, Rzany et al. 1993). Patients show ocular, buccal and genital mucosa involvement, with the probability of respiratory and gastrointestinal tract involvement.

The drugs which predominate in the pathogenesis of SJS-TEN are divided into two groups; those that provoke a hypersensitive response after short term administration and those that require a longer period to provoke a response (Roujeau, Kelly et al. 1995, Harr and French 2010).The former includes the antibiotics trimethoprim–sulphamethoxazole, sulphonamides, aminopenicillins, cephalosporins, quinolones, and chlormezanone, while the antiseizure drugs carbamazepine, phenytoin, phenobarbitone, and valproic acid, NSAIDs of the oxicam type, allopurinol, and corticosteroids all fall into the latter group (Roujeau, Kelly et al. 1995). Allopurinol has been identified as the drug

most commonly implicated in the development of SJS/TEN in Europe and Israel (Halevy, Ghislain et al. 2008) Infectious diseases have been shown to have a marked effect on SJS and TEN. This has been observed with HIV patients where the annual incidence is about 1,000 times higher when compared to the general population (Mittmann, Knowles et al. 2012) and with *M. pneumoniae* and *H. simplex* where SJS-TEN hypersensitivity reactions have been observed in rare cases without apparent drug use (Harr and French 2010).

Genetic associations have also been identified in the pathogenesis of these reactions and *this* has been demonstrated by the expression of HLA-B *15:02 alleles which had strong associations with carbamazepine induced SJS in Han Chinese. Their restriction to a particular ethnic grouping is indicative of an obvious relationship between ethnicity and SJS (Tassaneeyakul, Tiamkao et al. 2010, Chung and Hung 2012).

Provocation tests and skin testing are not considered with SJS-TEN patients because the risk of inducing a fresh episode or worsening an existing case is too high. Thus, clinical presentation and histology are currently the main diagnostic tools employed in SJS-TEN (Baldo and Pham, 2013). Primary treatment generally consists of discontinuation of the causative drug(s). Other modes of treatment which have been employed include the use of systemic corticosteroids and immunosuppressive agents such as cyclosporine and cyclophosphamide (Ghislain and Roujeau 2002). The use of intravenous immunoglobulins has been suggested but there is currently a lack of randomized control trials to assess the benefits and risks and to ultimately standardize the optimal treatment protocol (Momin 2009).

1.3 The immune system

The immune system is a consortium of biological structures and processes within an organism specifically designed for protection against disease. The ability to selectively detect a diverse range of pathogenic organisms remains a must for optimal functioning of this system. This is essential to enable the differentiation of self from pathogenic tissue.

The earliest mention of immunity was during the plague of Athens in 430 BC (Retief and Cilliers 1998). Later Louis Pasteur exploited Pierre-Louis Moreau de Maupertuis's findings of the 18th century to lay down the fundamental principles of vaccination (Plotkin 2005). Rapid advances were then made towards the end of the 19th century, and Paul Erlich was able to explain the specificity of the antigen-antibody reactions. This has formed the basis of a host of immunological assays to this date, a feat for which he was jointly awarded the Nobel Prize in Physiology or Medicine 1908 with Ilya Ilyich Mechnikov.

The two main components of the immune system are the innate immune system which is the first line of defence of the body against infection, and the more highly specialised adaptive immune system. All cells of the immune system originate in the bone marrow, but the differentiation of some may occur at a distant immunological site.

1.3.1 Innate immunity

This first line of defence is a part of the comprehensive immune system that consists of the cells and mechanisms responsible for the protection of the host from infection by other organisms, by eliciting their protective responses usually in a generic manner. This response is shared by both plant and animals (Janeway and Medzhitov 2002)

The innate immune system consists of anatomical barriers to infection which include epithelial surfaces that are impermeable to most pathogenic organisms. Skin exuviation, the peristaltic movement of the gastrointestinal tract, flushing action of tears and saliva are other mechanical ways employed to prevent infection. Chemically, fatty acids in sweat, lysozyme and phospholipase found in some secretions are also capable of inhibiting unwanted cell growth. If the anatomical barriers are unable to stop the progression of an infection, humoral barriers come into play in the form of an acute inflammatory reaction. This reaction is characterized by edema, the recruitment of phagocytic cells and the activation of the complement system. Cellular barriers to infection are evidenced by the involvement of polymorphonuclear cells as a part of the inflammatory response where they are recruited to the site of infection. Initial paradigms describe the non-specificity of the innate response. However some form of specificity is exerted via activation of a limited number of germline pattern-recognition receptors (PRRs) with the ability to recognize pathogen-associated molecular patterns (PAMPS) (Akira, Uematsu et al. 2006).

1.3.1.1 Cells of the innate immune system

(i) Macrophages. These are important phagocytic immune effector cells derived from monocytes**,** which also play vital homeostatic roles independent of their immune function (Mosser and Edwards 2008). Macrophages, sometimes referred to as 'professional phagocytes' are renowned for the uptake and degradation of infectious agents and senescent cells, participation in development, tissue remodeling, the immune response, and inflammation (Aderem and Underhill 1999).

Recent studies have demonstrated that resident macrophages are established during embryonic development and persist during adulthood, but independent of blood monocytes (Sawyer, Strausbauch et al. 1982, Volkman, Chang et al. 1983, Hashimoto, Chow et al. 2013, Yona, Kim et al. 2013, Epelman, Lavine et al. 2014). Though monocytes are able to enter steady-state non-lymphoid organs they recirculate to lymph nodes without differentiation to macrophages (Jakubzick, Gautier et al. 2013).

The molecular mechanisms underlying phagocytosis by macrophages is very complex due to the diversity of receptors and also the ability of some micro-organisms to influence their fate in unpredictable ways as they are internalized. In the midst of all this complexity a few shared features exist which include the initiation of particle interaction via interactions between specific surface receptors in the macrophage with ligands on the particle and the involvement of actin modelling (Aderem and Underhill 1999).

Agents capable of activating macrophages include components of the immunoglobulin and complement systems, products of activated lymphocytes and non-immunological agents. Studies on bacterial endotoxin-induced macrophage activation has suggested the involvement of various kinases like Protein kinase A, protein kinase C, MAPKs etc., and numerous inflammatory cytokines such as NF-kB, AP-1,TNF-α, IL-1, IL-6, IL-8, and IL-12 (Ogmundsdottir and Weir 1980, Klimpel 1996, Fujihara, Muroi et al. 2003). Macrophages have also been shown to present antigen to T cells in a primary response (Hume 2008).

(ii) Granulocytes

(a) Neutrophils. Neutrophils are the most abundant (40% to 75%) type of granulocyte in mammals. They are formed from stem cells in the bone marrow. They are polymorphonuclear in nature and form an essential part of the innate immune system. Like macrophages they are also considered as a form of professional phagocyte.

Neutrophils are able to interact with other cellular components specifically monocytes, dendritic cells, T and B lymphocytes in a bidirectional, multi-faceted manner (Nathan 2006).

They migrate towards a locus of infection, attracted by cytokines released by activated endothelium, mast cells, macrophages and T-cells, with TNF-α, GM-CSF, IL-8 and IFN-γ respectively, the culprit cytokines. Neutrophils also recruit and activate monocytes, dendritic cells (DCs) and lymphocytes via the release of cytokines with a resultant amplification of inflammation (Ear and McDonald 2008, Wright, Moots et al. 2010).

(b) Eosinophils. Similar to other granulocytes eosinophils undergo development and maturation in the bone marrow. They are found in low numbers constituting 1-4% of total peripheral blood (Shamri, Xenakis et al. 2011). They function as a bridge between the innate and adaptive immune system. Eosinophils secrete Th1 and Th2 cytokines (e.g. IL-4, IL-5, IL-9, IL-13, IL-12 and IFN-γ), acute proinflammatory cytokines (TNF-α, IL-1β), and immune inhibitory cytokines (e.g., TGF-β and IL-10). They also express complementary receptors for many of these cytokines (Lacy and Moqbel 2001, Spencer, Szela et al. 2009, Jacobsen, Helmers et al. 2012).

Migration to sites of inflammation is mediated by certain chemokines and leukotrienes. At these sites, eosinophils are then activated by cytokines released from Th2 cells (Shamri, Xenakis et al. 2011). The induction of eosinophils leads to the expression of major histocompatibility complex II (MHC-II) complexes and co-stimulatory molecules which are required for the functional activation of T-lymphocytes. Thus, eosinophils primarily viewed from their perspective as effectors of allergic responses and parasite elimination, also function as antigen presenting cells (Shi 2004, Padigel, Lee et al. 2006).

(c) Basophils. These are the least numerous of the granulocytes, and account for less than 1% of all peripheral blood leukocytes occurring in the human body with a half-life of a few days. Basophils arise and mature in bone marrow and when activated, degranulate to release histamine, proteoglycans, and proteolytic enzymes. IL-3 is the most predominant cytokine which drives the activation of basophils. IL-5, GM-CSF, histamine-releasing factor, and several chemokines are also able to prime basophils leading to enhanced IL-13 and IL-4 secretion, critical cytokines in the development of allergies and the production of IgE antibodies (Yamaguchi, Koketsu et al. 2009, Stone, Prussin et al. 2010).

(d) Dendritic cells (DCs). DCs are bone marrow derived cells which function as APCs and play a critical role in the regulation of the adaptive immune response. These cells exist as a complex, heterogeneous group of multifunctional APCs (Mohamadzadeh and Luftig 2004). DCs have been referred to as professional APCs because their primary role is the presentation of antigens, and the ability to induce a primary response in naïve T lymphocytes. To perform this function, DCs are capable of capturing antigens, processing them, and presenting them on the cell surface along with appropriate costimulatory molecules (Guermonprez, Valladeau et al. 2002). The role DCs have in the perpetuation of B cell function and recall responses has confirmed their importance in the initiation of immunological memory (Qin, Wu et al. 2000, El Shikh, El Sayed et al. 2010).

They are generally identified by their cellular size and morphology, with all DCs possessing the ability to activate T lymphocytes while manifesting distinct functions within each subset. DCs can be broadly classified as two major subsets: the inflammatory or infection-derived DCs, which develop from monocytes in response to stimulation, and the steady-state DCs, which are present at all times (Moore and Anderson 2013). Recent

convention has stratified DCs to be either myeloid or plasmacytoid. Myeloid DCs (mDCs) typically express myeloid antigens, while plasmacytoid DCs (pDCs) typically lack myeloid antigens and are distinguished by expression of CD123, CD303 and CD304. CD14+ DCs which are situated in tissues and lymph nodes are a third subset originally described as 'interstitial DCs' while the Langerhans cells (LCs), and microglia are two specialized self-renewing DC populations found in stratified squamous epithelium and parenchyma of the brain, respectively (Collin, McGovern et al. 2013).

(e) Natural killer cells (NK cells). NK cells are large granular lymphocytes that possess cytotoxic and effector functions. They make up 2% to 18% of total lymphocytes in human peripheral blood (Vivier, Tomasello et al. 2008). NK cells express several toll like receptors specifically, with studies showing the expression of TLR 1-8 and similar studies also show the expression of TLR 1-10 mRNA on purified human NK/NKT cells (Chalifour, Jeannin et al. 2004, Sivori, Falco et al. 2004, Lauzon, Mian et al. 2006). The magnitude and character of NK cell cytotoxic and cytokine responses is reliant on the cytokine milieu and on interactions with other cells of the immune system (Long 2007). IFN-γ, IL-12, IL-15, and IL-18 are all potent activators of NK cell function. Cells pre-activated with these cytokines *in-vitro* for 15 hours are detectable in high numbers 3 weeks after transfer into RAG-1$^{-/-}$ mice. The cells produce high levels of IFN-γ upon restimulation (Cooper, Elliott et al. 2009).

In addition to their cytotoxic and effector functions NK cells may act as regulatory cells having an influence on various cell types. For example immature DC killing by NK cells in humans and mice has been shown to influence homeostasis (Hayakawa, Screpanti et al. 2004, Vivier, Tomasello et al. 2008). Though little is known of the impact of NK cells in epithelia, a recent study employing a model of contact hypersensitivity has shown the

involvement of NK cells in memory type immune responses which are independent of T and B cells (O'Leary, Goodarzi et al. 2006).

1.3.1.2 PAMPs, DAMPs, MAMPs and sterile inflammation

Pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) are microbial and non-microbial signals respectively which are sensed by pattern recognition receptors (PRRs). The recognition of these signals leads to PRR activation and development of an acute inflammatory response. A third signal defined as microorganism-associated molecular patterns (MAMPs) from pathogens and commensal bacteria has also been suggested. This has been used in some instances to replace PAMPs due to the presence of similar molecules such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycans, lipoproteins and modulins which are recognized by PRRs and TLRs. A host of interactions have been observed to exist between these two groups which includes interaction manifested as binding between PAMPs and DAMPs exemplified by the binding of LPS from gram-negative bacteria (a PAMP) and several different DAMPs (Picard, Janela et al. 2010), the ability of DAMPs to act as PAMP transporters (Youn, Oh et al. 2008), occasional utilization of similar receptors such as the TLRs, and the PAMP induced release of DAMPs in infection (Baumann, Aspalter et al. 2010, Mills 2011, Escamilla-Tilch, Filio-Rodriguez et al. 2013).

The effector mechanisms underlying the inflammatory response ensures recruitment of leukocytes that are extremely effective at killing microbes but with a disadvantage of the production of highly reactive species which are also capable of destroying normal mammalian cells. DAMPs have been broadly characterized as self-molecules with the ability to activate inflammatory responses and mediate sterile inflammatory responses

(Matzinger 1994, Rubartelli, Lotze et al. 2013). Sterile inflammation refers to inflammation in the absence of microorganisms and as a result of trauma, ischaemia–reperfusion injury or chemically induced injury. Specific hallmarks of this response include the release of DAMPS which activate PRRs resulting in a marked recruitment to the injury site of neutrophils and macrophages and the enhanced secretion of pro-inflammatory cytokines specifically tumour necrosis factor (TNF) and interleukin-1 (IL-1). Sterile responses have also been found to occur in situations where IL-1 was lacking (Chen and Nunez 2010, Rock, Latz et al. 2010, Kubes and Mehal 2012).

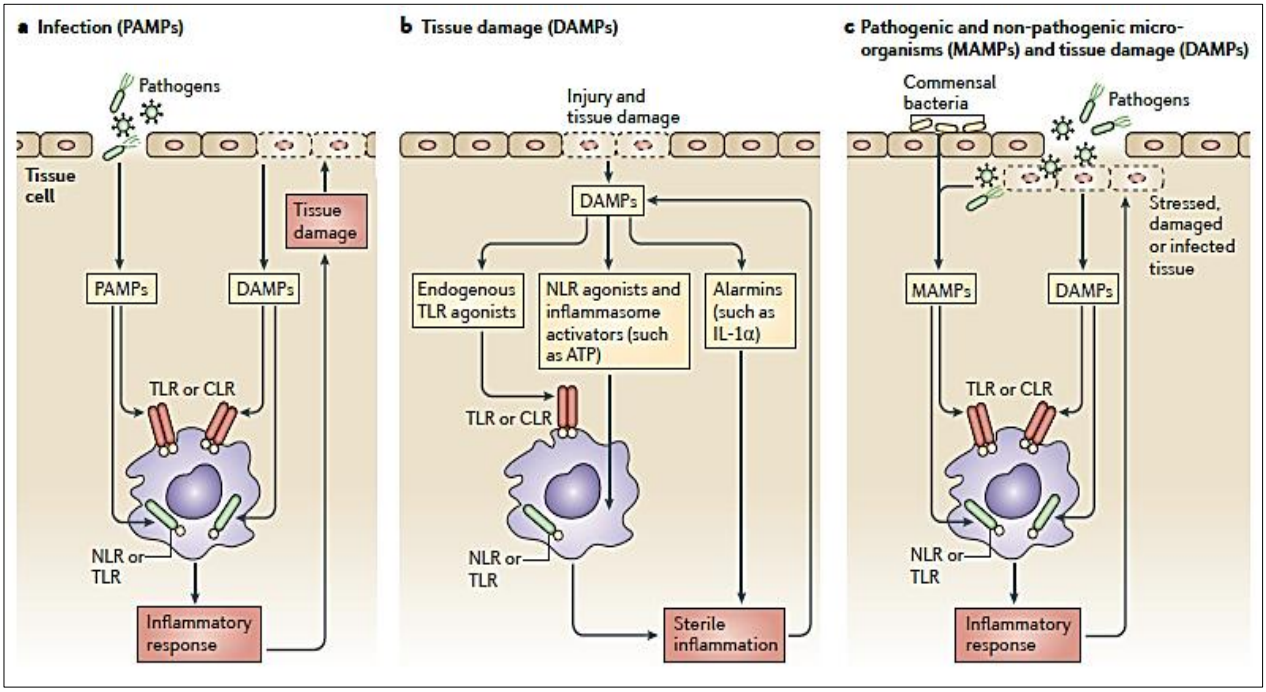


Figure 1.5. Inflammation as a result of PAMPs, DAMPs, and MAMPs. Danger signals elicited by PAMPs due to infection, DAMPs due to tissue damage and by MAMPs in collaboration with DAMPs due to pathogenic and non-pathogenic organisms with tissue damage (Adapted from Mills, K.H.; 2011).

Inflammation and the attendant fibrosis which arises due to sterile particle stimulation is associated with diseases such as gout, pseudogout, dementia due to Alzheimer's disease, and atherosclerosis (Akiba, Kehren et al. 2002, Liote 2003, Nuki and Simkin 2006, Beck, Morbach et al. 2009).

1.3.2 Adaptive immunity

The adaptive immune system, also referred to as the acquired immune system is a highly specific system composed of specialised cells and processes concerned with the protection of a host organism from pathogens.

Primarily mediated by antibodies secreted by B lymphocytes, and by T lymphocytes, the consequence of activation of the adaptive immune system is usually a direct effect on the pathogen. This may lead to bacteriolysis, opsonophagocytosis and killing, and viral neutralization by T lymphocytes (Robbins, Schneerson et al. 1996). Studies have shown innate immune system regulation via modification of the adaptive system. Examples include the interplay between NK and CD8+ T cells in mouse cytomegalovirus (MCMV) infection, and also in non-infectious uveitis (Mitrovic, Arapovic et al. 2012, Willermain, Rosenbaum et al. 2012)

A crucial feature of the adaptive immune system is the acquisition of an immunological memory which is specific to, and arises after an initial response to a given antigen. The memory acquired is long lived and ensures that in subsequent encounters with a similar pathogen the immune response is enhanced. The adaptive system includes both cell-mediated and humoral immunity components.

1.3.2.1 Cell-mediated immunity

(i) T Lymphocytes

T lymphocytes play a crucial role in cell-mediated immunity. They originate from pluripotent hematopoietic stem cells (HSC) located in the bone marrow, especially in the pelvis and iliac crest (Dzierzak and Speck 2008), and are so named due to their maturation in the thymus. The recognition of processed antigenic materials in the form of unique antigenic peptides presented by MHC on APCs occurs via the T cell receptor (TCR), thus conferring antigen specificity on T cells, though some receptors are able to ensure direct T cell recognition of the antigen without the recognised requirements for antigen processing and presentation (Bukowski, Morita et al. 1995, Morita, Beckman et al. 1995, Davis, Boniface et al. 1998, Bukowski, Morita et al. 1999, Wu, Groh et al. 2002).

The main subsets of T cells are the helpers, which express the CD4 co-receptor, and the cytotoxic T-cells, expressing the CD8 molecule (Mosmann, Cherwinski et al. 1986). A third subset, the regulatory T-cells (Tregs) has been identified. These cells express CD25 and FOXp3 markers (Sakaguchi, Sakaguchi et al. 1995, Fontenot, Gavin et al. 2003).

(a) CD4+ Helper T-cells: CD4+ lymphocytes, or helper T-cells, are a vital component of the acquired immune response. Their involvement in B-cell antibody production, enhancement of CD8 T-cell function and macrophage activation ensures their central role in maintaining the effectiveness of the acquired immune response (Bell and Westermann 2008, Zhu, Yamane et al. 2010). Furthermore they play an important role in the regulation of autoimmunity via immune suppression and the development of immunological memory. CD4+ antigen recognition occurs via presentation of antigenic peptides on MHC class II molecules by professional APCs. This is a precursor for the release of cytokines,

which don't possess any cytotoxic or phagocytic activity. T helper cells are essentially divided into two functional classes Th1 and Th2, and their corresponding activation ultimately determines the nature of pathogen clearance (Zhu and Paul 2008, Zhu, Yamane et al. 2010).

The Th1 cytokines of which IFN-γ is predominant, produce proinflammatory responses associated with the killing of intracellular parasites and perpetuation of autoimmune responses (Berger 2000).

The Th2 response is characterized by the production of various Th2 cytokines including IL-4, IL-5, IL-9, IL-10, and IL-13. Their functions include the activation of B cells to make neutralizing non-cytolytic antibodies, regulation of B cell class switching to IgE through their production of IL-4, and the control of tissue damage due to excessive Th1 proinflammatory responses (Berger 2000, Paul and Zhu 2010). After the resolution of the infection most of the CD4+ helper cells die off and a few remain as CD4+memory cells.

Newer T cell populations have been defined which include Th17, Th22 and Th9 subsets. Th17 cells are important in clearance of pathogens during infection and induction of tissue inflammation in autoimmune disease. They produce IL-17, IL-17F, and IL-22. IL-21 secretion by Th17 cells enhances their communication with the cells of the immune system (Korn, Bettelli et al. 2009). Th22 subset of human Th cells has been characterized by the secretion of IL-22 and TNF-α, but not IFN-γ, IL-4, or IL-17. These cells infiltrate the skin in individuals with inflammatory skin disorders and could be detrimental or protective in their action (Eyerich, Eyerich et al. 2009). The Th9 subset is the most recently described subset of T-helper cells. Characterized by their secretion of interleukin-9, which suggests their involvement in microbial immunity and immune mediated disease (Kaplan 2013). Th9 cells are found in the peripheral blood of allergic

patients, normal and inflamed skin and is released following specific antigen stimulation (Jones, Gregory et al. 2012, Purwar, Schlapbach et al. 2012, Cortelazzi, Campanini et al. 2013). The specific function of Th9 cells has been difficult to elucidate experimentally, thus establishing immune responses that are completely reliant on Th9 cells has not been possible.

(b) CD8+ cytotoxic T-lymphocytes: Cytotoxic T cells also referred to as killer T cell or cytotoxic T-lymphocytes (CTL) are a sub-group of T cells that are important mediators of adaptive immunity. They induce the death of cells infected with viral, bacterial, and certain protozoan pathogens. Naïve cytotoxic T cell activation occurs when antigenic derived peptides (8-10 amino acids in length) bound to MHC I on antigen presenting cells are presented to CD8+ cell bearing TCR specific for that antigen. This leads to clonal expansion and migration of activated cytotoxic lymphocytes to different body compartments in search of the specific MHC class I peptide. Antigen presenting cells such as dendritic cells are also capable of inducing naïve T cell proliferation and differentiation in response to an antigen due to their expression of co stimulatory molecules (Germain 1994, Banchereau and Steinman 1998, Harty, Tvinnereim et al. 2000). Similar to what occurs with $CD4^+$ helper T cells, upon the resolution of the infection most $CD8^+$ cells die and a few are retained as long-lived memory cells.

(c) Regulatory T Cells (Tregs). Tregs are a sub-group of T cells that are actively engaged in the maintenance of immunological self-tolerance and immune homeostasis by the suppression of effector T cells and other immune responses (Sakaguchi 2004). Treg suppressive effects also extends to immune responses involving B cells, NK cells, NK T cells , monocytes as well as dendritic cells (DCs) and various cancers (Schmidt, Oberle et al. 2012). CD25 and FoxP3 have been identified as phenotypic markers for suppressive

CD4+ T cells in naïve mice, but also humans within the $CD4^{+}CD25^{high}Foxp3$ T cell population (Sakaguchi, Sakaguchi et al. 1995, Baecher-Allan, Brown et al. 2001, Fontenot, Gavin et al. 2003, Yagi, Nomura et al. 2004, Schmidt, Oberle et al. 2012). A role for the co inhibitory molecule CTLA-4 expressed in human and murine Tregs, in the suppressive effects of Tregs has been mooted due to the fact that an inhibition or lack of in mice, leads to the development of spontaneous autoimmunity which is mitigated by the presence of Tregs (Bachmann, Kohler et al. 1999, Read, Malmstrom et al. 2000, Takahashi, Tagami et al. 2000).

1.3.2.2 The humoral immune system

Humoral immune responses are mediated via antibodies produced by B-lymphocytes. These antibodies are found in the fluid component of blood, or plasma and in extracellular fluids classically referred to as ''humors''. Hence the birth of the term 'humoral immunity'.

1.3.2.3 B-cell development and differentiation

B lymphocytes develop in the bone marrow from hematopoietic precursor cells, in adult human subjects and in all mammals. Early development is characterized by the ordered rearrangement of the Ig H (heavy) and L (light) chain loci, occurring via the functional reconstruction of their respective immunoglobulin gene segments which are the V, D and J for the heavy chain, and V and J for the light chain. The diverse repertoire of functional VDJ_H and VJ_L generated encode the B-cell receptor (BCR), potentially conferring onto the BCR an ability to express antibodies capable of recognizing more than 5×10^{13} different antigens (Brack, Hirama et al. 1978, LeBien and Tedder 2008, Pieper, Grimbacher et al.

2013). These are the earliest forms of B cells and are referred to as pro-B cells. At this stage which is the earliest in B cell development they are thought to express $CD34^{+}CD10^{+}CD19^{-}$ common lymphoid progenitors (CLP) before they mature via $CD34^{+}CD19^{+}CD10^{+}$pro-B. Similar populations have been defined as $CD34^{+}CD38^{+}CD10^{+}CD19^{-}$ but also with the expression of CD117 (LeBien 2000, Blom and Spits 2006, Hystad, Myklebust et al. 2007).

Pre-B cells arise from pro-B cells and are formed by the pairing of surrogate L chains (SLC) with the resultant cytoplasmic expression of μ H chain (LeBien and Tedder 2008). These cells with a functional μ are referred to as immature B cells and express cell surface IgD, IgM as well as CD21 and CD22 (Cuss, Avery et al. 2006, Cariappa, Chase et al. 2007, Alomar 2014). It should also be noted that this developmental process is loaded with checkpoints, which serve to censor autoreactive cells, employing various mechanisms such as clonal deletion, BCR editing or by putting implicated cells in a state of anergy (Hartley, Crosbie et al. 1991, Gay, Saunders et al. 1993). Studies suggest that this selection against self-reactive B cells is exclusive to the pro/pre-B cell stages and very unlikely at the latter stages of B cell development (Tussiwand, Bosco et al. 2009).

Following positive selection, the migration of immature B cells to the spleen occurs where they are referred to as transitional B cells which express CD93 and CD19 clusters (Tussiwand, Bosco et al. 2009). Transitional B cells develop further into a heterogeneous population of mature B cells, which include re-circulating cells in the B-lymphoid follicles in the spleen and lymph nodes and non-circulating cells which sequester in the marginal zone (MZ) of the spleen (Martin and Kearney 2002). Signalling via the BCR, Notch-2, the BAFF receptor and other signals including that from the NF-κB pathway, all play roles in

determining the post transitional B cell fate into either follicular or marginal zone B-cells (Pillai and Cariappa 2009).

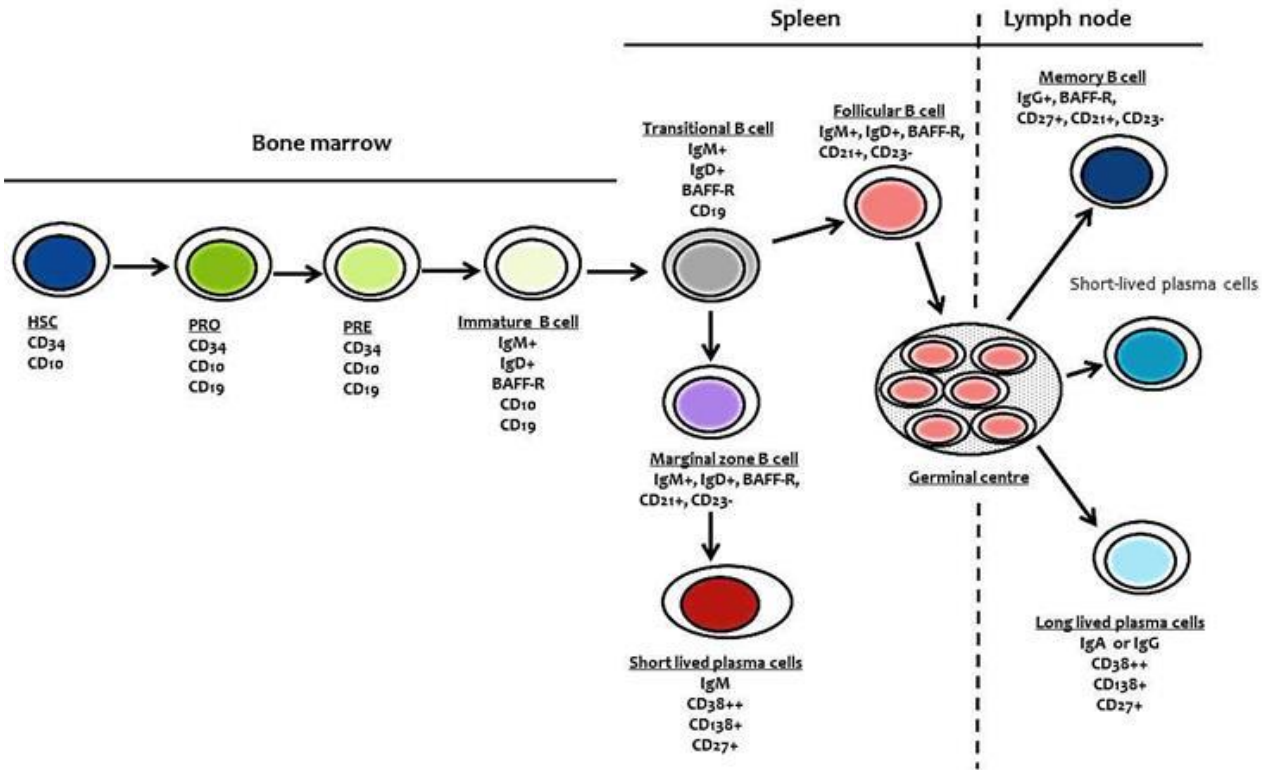


Figure 1.6. B cell development from haematopoietic stem cells (HSC).

B lymphocytes may serve either as antigen presenting cells (APCs) or produce antibodies to prevent infection. As an APC, their activation due to the binding of a ligand to the BCR leads to a cascade of intracellular signalling which results in the internalization, and processing of the antigen and subsequent presentation of antigenic peptides on MHC class II to T cells (Lanzavecchia 1985, Treanor 2012).

The close proximity of the T cell zones to the follicles also allows for the interaction between activated B- and T- cells. Thus, the migration to T cell zones of secondary lymphoid tissues allows for the recruitment of T cell help from affiliated T cells that have been previously activated. Hence follicular B cells are the ideal candidates for T cell-

dependent immune responses to protein antigens, but are also able to respond in a T cell-independent manner to blood borne pathogens (Cariappa, Mazo et al. 2005, Cariappa, Chase et al. 2007, Pillai and Cariappa 2009).

The interaction between B cells and T cells, leads to differentiation along either follicular or extrafollicular pathways. In the extrafollicular pathway short-lived plasmablasts only are formed by B cells. These plasmablasts though short-lived are able to provide critical functions, such as antibodies that protect against infection, even though the antibodies express only low affinity for the antigen (Luther, Maillard et al. 1997, MacLennan, Toellner et al. 2003, Lee, Rigby et al. 2011). The follicular pathway sees the formation of the germinal center by activated B cells. Here, B-cells undergo certain cellular events which include clonal expansion characterized by proliferation and amplification of the B cell; mutation of the heavy and light chain genes leading to an enhanced diversity in the antibody pool, by a process termed somatic hypermutation; and selection.

The resulting B cells consequently leave as high affinity long-lived plasma cells or memory B-cells (MacLennan 1994). Antigen driven production of high affinity antibodies (affinity maturation) follows a similar pattern and starts with the light and heavy chains (V_L and V_H genes) selected in the primary response. Following the somatic hypermutation of the V genes, clonal selection of B cells that produce antibodies of the highest affinity are then selected. This has been confirmed by recent advances in phage-display technology which has been able to mimic this process *in-vitro* (Fujii 2004).

Isotype or class switching is a biological process occurring after activation of the B cell, which allows the cell to produce different classes of antibody (IgA, IgE, or IgG) (Market and Papavasiliou 2003). The different classes of antibody, and thus effector functions, are defined by the constant (C) regions of the immunoglobulin heavy chain.

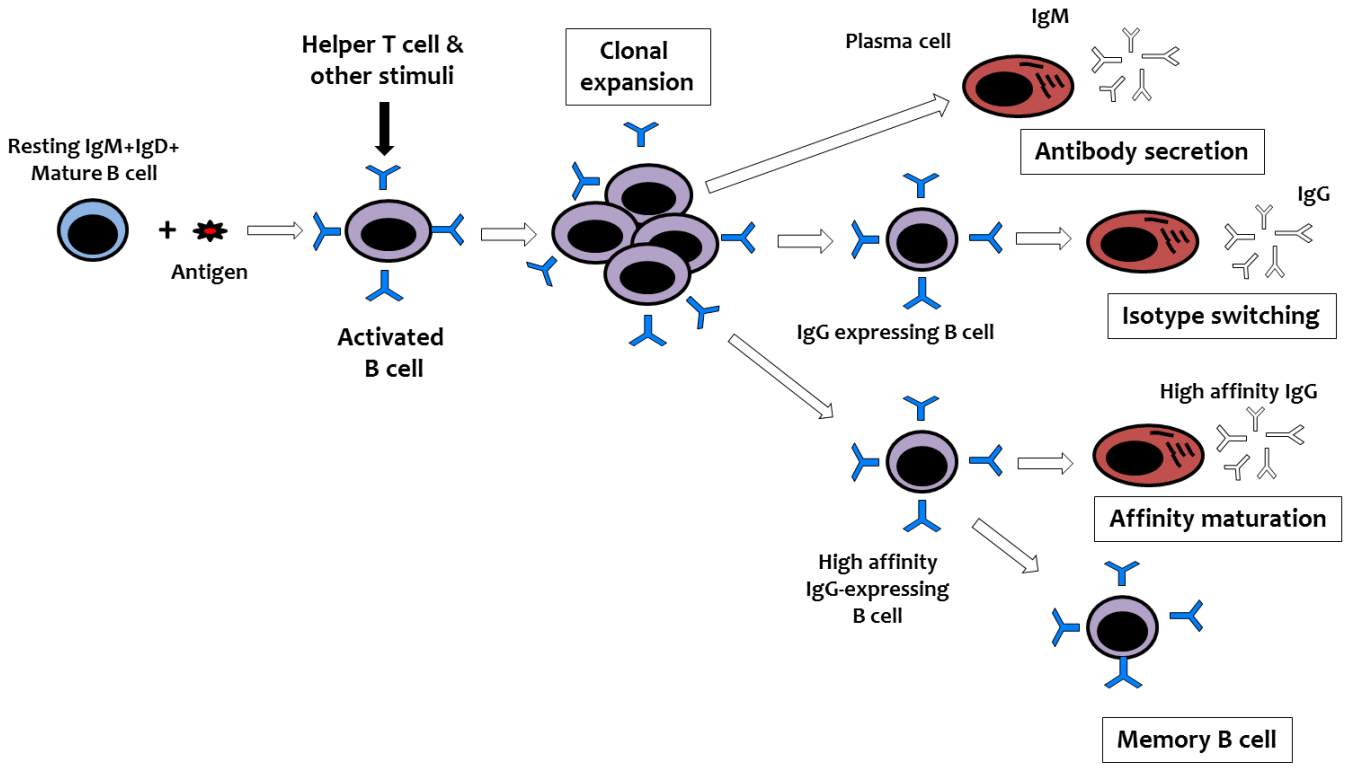


Figure 1.7. B cell proliferation in response to antigens and T cell help. Figure adapted from Sridhar Rao P.N, 2014.

1.3.2.4 Immunoglobulin secretion

The discovery in 1890 by Von Behring and Kitasato (von Behring and Kitasato 1991) of a substance in serum from rabbits immunized with tetanus toxin that had the ability to neutralise the 'poison of tetanus' when transferred to healthy rabbits, was the first of many studies showing the presence of immunoglobulins also referred to as antibodies in animals and humans (Schroeder and Cavacini 2010, Novaretti and Dinardo 2011).

Secreted by plasma cells immunoglobulins can be expressed either as a membrane bound form which is attached to the B cell surface and hereafter referred to as the B-cell receptor (BCR) or secreted as a soluble antibody which is released into the blood tissues

to combat invading microorganisms (Parker 1993, Borghesi and Milcarek 2006). They are heterodimeric proteins which are made up of two identical light chains and 2 identical heavy chains which are composed of two functional units created by series of gene arrangements; a variable domain for antigen binding and a constant domain for specific effector functions (Schroeder and Cavacini 2010).

The antibody is composed of the Fab (Fragment antigen-binding) region which is responsible for the recognition of specific foreign objects. This region is made up of one variable and one constant domain from each of the heavy and light chains. The Fc (Fragment crystallizable) region is responsible for the modulation of the effector mechanisms of the antibody.

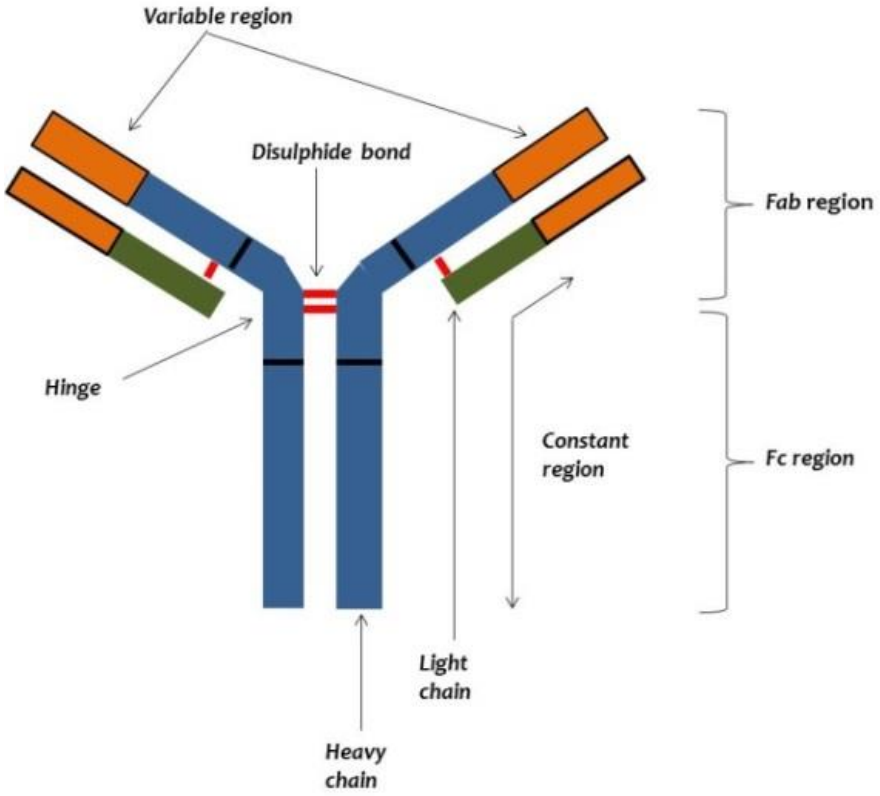


Figure 1.8. Basic structure of the antibody.

Depending on the class of the antibody the 2 heavy chains which make up the Fc region can contribute constant domains. This ensures that the antibody is able to generate the appropriate immune response for antigens by binding to a specific class of Fc receptors, and complement proteins. This leads to the regulation of effects such as recognition of opsonized particles and a host of other physiological effects (Woof and Burton 2004).

1.3.2.5 Immunoglobulin classes

Antibodies are generally classified in mammals into IgA, IgD, IgE, IgG and IgM, and are referred to as isotypes or classes. These isotypes have diverse biological functions, locations and ability to deal with distinct antigens.

Out of all the immunoglobulins and IgG subclasses, special attention has been paid to the role of IgG4 in the pathogenesis of disease which has assumed a new dimension. A plethora of diseases termed IgG4-related diseases (IgG4-RD) are identified and have been described as systemic immune mediated diseases. These diseases could involve any organ, their common denominator being the obvious IgG4 involvement (Brito-Zeron, Ramos-Casals et al. 2014). IgG4-RD have been identified and include sclerosing pancreatis, sclerosing cholangitis suggesting biliary tree involvement, thyroiditis, IgG4 related pulmonary disease, Mikulicz's disease and neurologic disease amongst others (Hamano, Kawa et al. 2001, Kamisawa, Nakajima et al. 2006, Inoue, Zen et al. 2009, Takahashi, Yamamoto et al. 2012, Flanagan, Chowdhary et al. 2014, Poplawska-Kita, Kosciuszko-Zdrodowska et al. 2015)

Table 1.2. Table of IgG classes. The table shows their structures, anatomical locations and functions.

Class	Subclass	Structure	Anatomical location and function
IgA	**IgA1, IgA2**	Dimeric	Produced locally at mucosal sites, such as the gut, respiratory tract and urogenital tract. Found in colostrum (Woof and Kerr 2004, Woof and Russell 2011).
IgD	**1**	Monomeric	IgD is expressed on the surface of the majority of mature B cells before antigenic stimulation. IgD is also secreted (Finkelman, van Boxel et al. 1976, Finkelman, Woods et al. 1979, Chen and Cerutti 2011). Functions as an antigen receptor on B cells prior to antigen exposure (Roes and Rajewsky 1993, Geisberger, Lamers et al. 2006).
IgE	**1**	Monomeric	Binds to allergens and triggers histamine release from mast cells and basophils, and is involved in allergy (Schroeder, MacGlashan et al. 2001). Also protects against parasitic worms (Bell 1996, Fitzsimmons, Falcone et al. 2014).
IgG	**IgG1,IgG2, IgG3, IgG4**	Monomeric	Most abundant antibody in the blood. Functions include opsonization, activation of the classical pathway of the complement system, the neutralization of toxins. Antibody-dependent cell-mediated cytotoxicity and intracellular antibody-mediated proteolysis (Janeway, Travers et al. 2001).
IgM	**1**	Pentameric	Produced in the spleen and expressed on the surface of B cells and in a secreted form. Possesses very high avidity and effective at complement activation.

1.4 Cystic fibrosis (CF)

CF is a lethal genetic autosomal recessive condition that leads to abnormal airway epithelial ion transport (Whitaker, Meng et al. 2011). The majority of the incidents of morbidity and mortality in patients have been attributed to pulmonary disease, with respiratory failure accounting for deaths in 85% of patients (Flume, O'Sullivan et al. 2007).

CF is distinct in its racial, ethnic and geographic distribution. The prevalence of CF has been found to be 1/1800 to 1/5000 Caucasians in Europe, United States and Canada, 1/14000 in Afro-Americans, 1/4000 in Finland and 1/7,000 in Brazil. CF exists in all known ethnic groups though rare in Africans and Asians (Brice, Jarrett et al. 2007, Haack, Aragao et al. 2013).

1.4.1 Genetic determinants and management of CF

The CFTR gene which is associated with CF is responsible for encoding the CF Transmembrane Conductance Regulator (CFTR) which works as a channel for the transport of chloride ions across the apical membranes of epithelial cells. Even though many mutations of this gene have been discovered, with about 2000 variations being shown to exist (US Cystic Fibrosis Foundation), very few have actually shown the tendency to cause CF. Previous studies have shown that on average, 10 mutations account for 79.7% of alleles in patients with CF but about 70% of European patients exhibit a similar defect, the F508del which leads to the deletion of 3 bases and eventual loss of phenylalanine. Two other common mutations in the European population are the G542X and N1303K (Estivill, Bancells et al. 1997, Bobadilla, Macek et al. 2002, Grossman and Grossman 2005, Castellani, Cuppens et al. 2008).

A defect in the CF gene leads to the abnormal chloride conductance across the apical membrane of the epithelial cell. The resultant airway surface liquid depletion promotes ciliary collapse and inhibition of mucociliary transportation thus providing a suitable medium for the microbial colonisation with infection and inflammation as the end result (Ratjen 2009). A fallout of this process is the development of increased numbers of membrane asialoGM1 molecules, which are thought to promote the increased binding of

Pseudomonas aeruginosa and *Staphylococcus aureus* (Saiman and Prince 1993), microorganisms which have been identified to predominate in cases of respiratory infections in these patients. However, a more recent elucidation of the crystal structure of *Pseudomonas aeruginosa* has found this proposed view to be inconsistent with the steric realities. This is due to the fact that the C-terminal, which is the proposed binding site of *P. aeruginosa,* is poorly positioned (Hazes, Sastry et al. 2000, Schroeder, Zaidi et al. 2001).

Due to the presentation of symptoms which are specific to patients with CF such as the development of polyps which have the potential to activate sinus infection, patients usually require a longer and more intensive antibiotic treatment, than with patients who present solely with pneumonia (Frederiksen, Koch et al. 1997). ß-Lactam antibiotics are the corner stone for the management for patients with CF. They are generally employed to prevent, eradicate or control infections which arise due to bacterial colonisation in the upper respiratory tract. Commonly used beta lactams include intravenous aztreonam, ceftazidime, flucloxacillin, meropenem, piperacillin-tazobactam, and Ticarcillin-clavulanic acid (CF trust 2009).

Unfortunately their use is restricted due to the occurrence of severe hypersensitivity reactions, which are delayed in nature. Several studies involving patients with CF have reported a high incidence of reactions due to certain ß-lactam antibiotics, with frequent manifestations of drug-induced fever (DIF) being reported most commonly with piperacillin. Typically, the incidence of DIF in patients with CF (2.6 to 32%) is greater than that reported in non-CF patients (generally < 5%) (Pleasants, Walker et al. 1994). This trend corresponds with the incidence of β-lactam hypersensitivity in the general

population of about 1-8% (Parmar and Nasser 2005) and 30-50% in patients with CF (Lin 1992).

1.5 Immune mechanisms of drug-induced allergy

It is important to note that the immune mechanisms that are highlighted in this section are primarily concerned with allergic reactions mediated by T cells. The recognition of conventional antigens by T cells is made possible due to specialized antigen presenting cells (APCs), such as dendritic cells, macrophages, and B lymphocytes, which capture extracellular protein antigens, internalize and process them, then display the processed antigen on the surface of major histocompatibility complex (MHC) molecules. CD4+ helper T lymphocytes are thought to recognize antigens in association with MHC class II molecules (MHC class II restricted recognition), and CD8+ cytotoxic lymphocytes (CTLs) recognize antigens in association with class I molecules (MHC class I restricted recognition). Dendritic cells are the most efficient APCs for initiating primary responses by activating naive T-cells. Macrophages and B lymphocytes present antigens to differentiated helper T-cells in the effector phase of cell-mediated immunity and in humoral immune responses, respectively.

The mechanisms by which a chemical or drug acts as an antigen and elicits immune activation has not been fully elucidated (Schnyder and Pichler 2009). Most pharmaceutical agents cannot bind directly to MHC molecules due to their low molecular weight and therefore cannot be directly presented to T cells by APCs (Hausmann, Schnyder et al. 2012), but drug-specific T-cells do exist. The hapten and pro-hapten concept, P-I concept, and the altered self-peptide repertoire are theories that have been

proposed to try and explain how drugs can act as antigens for T cells (Adam, Pichler et al. 2011).

1.5.1 Hapten concept/Pro-hapten concept

Haptens are low molecular weight compounds (MW < 1000 Daltons), that may only elicit an immune response when complexed covalently to a large carrier such as a protein. "Hapten" is a term coined by Landsteiner and Jacobs to explain both humoral and cellular immune responses to low molecular weight chemicals observed in their research (Landsteiner and Jacobs 1935, Chipinda, Hettick et al. 2011, Pichler, Naisbitt et al. 2011). Most drugs being of low molecular weight were thought to be unable to stimulate immune responses due to their size, and thus undergo haptenation (Naisbitt, Hough et al. 1999, Naisbitt, Gordon et al. 2001, Naisbitt, Farrell et al. 2002) by binding to endogenous/self-proteins. This leads to modification of the proteins, and confers on them a form of immune recognition. Chemical haptens or drugs also have the tendency to bind covalently to particular amino acid residues with some β-lactams and their derivatives as examples, binding covalently to lysine residues of human serum albumin (Whitaker, Meng et al. 2011, El-Ghaiesh, Monshi et al. 2012). The pro-hapten concept would require bio-activation/metabolism of drugs or chemical agents to intermediates that can react with endogenous constituents to initiate a chain of chemical and biochemical events which could lead to cellular damage. Nitroso sulfamethoxazole (SMX-NO), the protein reactive metabolite of sulfamethoxazole (SMX) has been found to selectively modify cysteine residues on proteins. The abiotic activation of drugs and chemical agents without the requirement of a particular enzymatic system is also possible. Following exposure to air, various non-reactive molecules such as abietic acid (a

colophony resin), terpenes and p-phenylenediamine (PPD) are rapidly transformed into hapten reactive derivatives. Thus, in addition to the hapten and pro-hapten mechanisms of activation a further extension of the nomenclature was proposed. The term 'pre-hapten' was therefore coined to include nonreactive sensitizing chemicals that undergo activation as described above (Matura, Skold et al. 2005, Lepoittevin 2006).

Previous studies have also established a relationship between the nature and magnitude of the T-cell response and the degree of covalent hapten binding to proteins. In that study dinitrochlorobenzene (DNCB) and dinitrofluorobenzene (DNFB), chemicals that exhibited a Type 1 cytokine secretory profile were shown to bind selectively to cellular proteins while trimellitic anhydride (TMA), fluorescein isothiocyanate (FITC) and dinitrobenzenesulfonyl chloride (DNBSCI) that exhibited type 2 secretory profiles bound to serum. Notably, the reactions were not associated with the chemical structures or known amino acid specificity (Hopkins, Naisbitt et al. 2005).

1.5.2 The P-i concept

The direct pharmacological interaction of drugs with the immune receptors referred to as the P-i concept proposes that some drugs may bind non-covalently to some of the variable antigen specific T cell receptors (TCR's) and MHC molecules (Gerber and Pichler 2004). This concept tries to explain the induction of a primary immune response without the initial sensitization from previous exposure by the drug allergen. Though the exact mechanism of this TCR dependent T-cell activation by drugs has not been elucidated. Three ideal properties which a T cell must possess for activation to take place have been defined which include the expression of a TCR that may bind the drug, a low threshold of

activation and an additional interaction of the TCR with the MHC on the APC which must occur to enhance the response of the drug. (Schnyder and Pichler 2009).

1.5.3 Altered Self-peptide repertoire:

MHC molecules are highly polymorphic proteins encoded by Human leukocyte antigens (HLAs) that initiate immunity by presenting pathogen-derived peptides to T cells. Abacavir and carbamazepine are two drugs that mediate significant immune hypersensitivity reactions that are strongly associated with the HLA alleles B*57:01 and B*15:02 respectively (Illing, Vivian et al. 2012, Ostrov, Grant et al. 2012). These reactions are not explained simply by either the hapten and pro-hapten or the P-i concepts of drug binding. What we now know is that abacavir and carbamazepine (to a lesser extent) are able to bind specifically to the HLA alleles, altering the shape and chemistry of the antigen-binding cleft, and are thus able to influence a change in the pool of presented self-peptides capable of binding the HLA alleles B*57:01 and B*15:02 (Illing, Vivian et al. 2012). It is this guided alteration of endogenous peptides that is assumed to be responsible for the significant incidence of hypersensitivity reactions in patients bearing the HLA risk alleles. More recently a similar association has been reported with acyclovir and HLA-B*57:01 but without the induction of hypersensitivity (Metushi, Wriston et al. 2015).

1.6 Functions of B-cells in disease states

The involvement of the immune system in the pathogenesis of many human diseases, for example auto- and acquired immune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), hypersensitivity reactions, HIV-AIDS have been studied

in detail. In trying to decipher the mechanisms and functional roles played by the different components underlying the immune response seen in these conditions, the emphasis has been skewed in favour of the role T lymphocytes play. This was due to the popularly held view that T cells comprise the fundamental pathological component of autoimmune diseases, and the autoantibodies identified therein considered as accessories secondary to the loss of T cell tolerance. More recent studies have led to the development of B cell directed therapy. This has generated substantial interest in the potential modulatory role B cells may play in the management of such conditions (Lopes-Carvalho and Kearney 2005, Browning 2006).

Some conditions may involve interplay between the B cells and other immune components such as in RA, where alongside rheumatoid factors (RF) and anticitrullated antibodies generated by B cells which promote immune complex formation and complement activation, there is also the reactivation of autoreactive T cells by the B cells acting as APCs (Dorner and Burmester 2003, Silverman and Carson 2003). Other autoimmune disorders such as SLE are derived from the generation of pathogenic autoantibodies. Immunodeficiency manifested as chronic inflammation has been found to involve B cells (Pelton and Denman 1982, Nashi, Wang et al. 2010). The efficacy of B cell depletion therapy in RA patients employing Rituximab an anti-CD20 chimeric monoclonal antibody is a perfect illustration of the relevance of B cells in autoimmune disease. A significant reduction in autoantibody levels, and significant improvement in clinical symptoms, being clear indicators (Edwards, Leandro et al. 2004, Edwards, Szczepanski et al. 2004, Lim, Beers et al. 2010). It should be noted though that responses obtained with SLE patients were highly variable, but in general this class of agents shows a lot of promise (Pescovitz 2006, Mandik-Nayak, Ridge et al. 2008). Studies have shown CD19 B

cells to play important roles in the elicitation of contact hypersensitivity (CHS), a cutaneous immune reaction mediated mainly by antigen specific effector T-cells. The mechanism of their action is probably via the antigen-specific IgM dependent recruitment of effector T cells, function of regulatory B-cells (Bregs), and also the manifestation of defective CHS responses elicited by B cell deficient mice (Tsuji, Szczepanik et al. 2002, Watanabe, Fujimoto et al. 2007).

Though the immunoglobulin subclasses are all closely related, they have different heavy chain structures and effector functions. The Fc portion of the IgG molecule (see figure 1.8) contains binding sites for complement (C1q), IgG-Fc receptors (FcγR) on effector cells and neonatal Fc receptor (FcRn). The subclasses of IgG which include IgG1, IgG2, IgG3 and IgG4 share a 90% homology but still possess unique profiles with respect to effector function. The individuality in effector function exists mainly due to the structural variations in the hinge regions (Hamilton 1987) and amino acid differences found in the N-terminal CH2 domain though other factors have been postulated (Hovenden, Hubbard et al. 2013, Theo and Gestur 2014). These variations give rise to the differential binding of IgG subclasses to the C1q and FcγR sites which in turn also determines the elicitation of a number of effector functions.

The regulatory function of IgG4 has been observed in the control of IgE-mediated reactivity. Tolerance has been observed in human filariasis where antigen specific IgG4 acts as a blocking antibody with an increase in circulating levels associated with tolerance (Hussain, Poindexter et al. 1992). Elevated levels of specific IgG4 was also observed in immunotherapy with bee venom (Wyss, Scheitlin et al. 1993) and in the maintenance of tolerance to cow milk in atopic individuals (Ruiter, Knol et al. 2007). The role of IgG4 in

the regulation of tolerance in cellular immune mediated hypersensitivity reactions beyond already elucidated mechanisms of microbial opsonization prior to processing and presentation still remains unclear.

1.7 Evidence of humoral involvement in hypersensitivity reactions

Historically the immune system has been found to be involved in the development of ADRs, with effects ranging from those due to immunosuppression (Salvadori and Bertoni 2003, Stucker and Ackermann 2011) to severe hypersensitive reactions discussed earlier in this text.

The characterisation of drug-specific CD4+ T-cell clones coupled with the identification of secreted cytokine products from these cells highlights their role in the elicitation of certain forms hypersensitivity reactions (Akiba, Kehren et al. 2002, Naisbitt, Farrell et al. 2003, Heimann, Janda et al. 2011, El-Ghaiesh, Monshi et al. 2012). The identification of anti-drug antibodies (Coleman, Yeung et al. 1986, Bougie, Wilker et al. 2006), immunoglobulin subclasses (Eric et.al, 2009), their secretions indicates that the humoral immune system contributes to the effector and regulatory immune process in the disease pathogenesis (Lund 2008, den Reijer, Lemmens-den Toom et al. 2013).

Antidrug antibodies have been identified previously with studies from the 50's and 60's to the present day focussing on the detection and characterization, their hypersensitivity mechanisms, clinical implications and the development of improved detection techniques. Several studies are listed in table 1.3.

Table 1.3. Detection of antidrug antibodies (studies from 1961 – 2015). Emphasis is on the detection and characterization, mechanisms, clinical implications and experimental detection of antidrug antibodies.

	Study focus	
1.	Detection and characterization	(de Haan, Boorsma et al. 1979, Christie, Coleman et al. 1988, Christie, Breckenridge et al. 1989)
2.	Mechanisms of hypersensitivity	(Levine and Ovary 1961, Chandra, Joglekar et al. 1980)
3.	Clinical implications	(Okuno and Crockatt 1976, Mok, van der Kleij et al. 2013, Lundkvist Ryner, Farrell et al. 2014)
4.	Assay development for antidrug antibody detection	(Van Cleave 2003, Bourdage, Cook et al. 2007, Macy, Goldberg et al. 2010, Bloem, van Leeuwen et al. 2015)

Although a rise in the numbers of B and T-cells coincides with the protection against re-infection with blood borne pathogens, it is also dependent to a large extent on pre-existing titres of neutralizing antibodies. It has been hypothesized that antigen dependence via various cellular mechanisms, and the repetitive stimulation of B cells which may occur via a chronic low-grade infection or repeated exposure to external virus, bacteria or toxins is required for the differentiation of memory B cells to short-lived plasma cells with the ability to maintain high antibody titres (Ochsenbein, Pinschewer et al. 2000).

1.8 Covalent binding in drug-specific B-cell activation

The covalent binding of drugs to proteins leads to modifications that result in the

production of major and minor antigenic determinants. Major determinants include penicilloyl and nitroso metabolites from penicillin, and sulfamethoxazole respectively, of which multiple experimental data exists to show their interactions with the cellular adaptive system (Green, Rosenblum et al. 1977, Spath, Huber et al. 1979, Naisbitt, Hough et al. 1999, Naisbitt, Gordon et al. 2001). The minor antigenic determinants on the other hand may contribute to the induction of IgE mediated allergic responses (Weltzien and Padovan 1998). Mass spectrometry has significantly aided the qualitative and quantitative analysis of these peptides, proteins, reactive metabolites and conjugates. Initially developed in the early 20th century to measure masses of atoms by physicists, the application has evolved to natural products identification by scientists to their primary application under the purview of analytical chemistry in the latter part of the 20th century. The development of plasma desorption, fast atom bombardment (FAB), and thermospray ionisation were steps in the identification of proteins but were faced with the disadvantages of using high concentrations of small proteins and the fact they didn't work well with larger proteins (Macfarlane and Torgerson 1976, Barber, Bordoli et al. 1981, Morris, Panico et al. 1981, Liberato, Fenselau et al. 1983, Vestal and Blakely 1983, Griffiths 2008). Later development of matrix assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI) techniques with enhanced utility were the keys required to unlock the doors enabling the full characterization of these proteins and peptides (Karas and Hillenkamp 1988, Tanaka, Waki et al. 1988, Fenn, Mann et al. 1989). Thus it was suggested that these methods possess the capability to define the role of covalent binding of drugs and other low molecular weight chemical compounds to proteins in immunogenicity (Weltzien and Padovan 1998). Current applications involve the use of Multidimensional liquid chromatography with ESI (MudPIT) to characterize cisplatin

binding sites (Will, Wolters et al. 2008), liquid chromatography-mass spectrometry (LC-MS) analysis to identify 2,4-dinitro-1-chlorobenzene covalent adducts in the skin and LC-MS/MS to detect the presence of acyl glucuronide metabolites in diclofenac patients (Aleksic, Pease et al. 2008, Hammond, Meng et al. 2014). Other applications involved defining protein haptenation mechanisms in contact and delayed-type drug hypersensitivity (Aleksic, Pease et al. 2007, Meng, Jenkins et al. 2011, Jenkins, Yaseen et al. 2013).

1.9 Immune response in β-lactam hypersensitivity

Utilizing basic immunological principles, researchers have developed various methods which have been employed in the detection of the immune response in drug hypersensitivity. Hence the fact an infection gives rise to a significant increase in the number of lymphocytes specific for the target pathogen (Datta and Sarvetnick 2009), has led to the use of proliferation assays specifically the lymphocyte transformation test (which we shall discuss further in a later chapter) for the confirmation of hypersensitivity to chemical agent/drugs molecules/haptens. The generation of T-cell clones (Naisbitt, Farrell et al. 2003, Naisbitt, Farrell et al. 2005, Castrejon, Berry et al. 2010), the use of flow cytometry (Quah and Parish 2012) and the application of the ELISpot and enzyme linked assays have all contributed to enhance our understanding of the molecular processes which underlie drug hypersensitivity (Pichler and Tilch 2004, El-Ghaiesh, Monshi et al. 2012).

1.10 Aims and objectives

Having recently described the cellular immunological processes that underlie drug-specific responses in hypersensitive patients: I would like to analyse in depth, the involvement of the humoral immune system which has been left somewhat ignored. I have specifically focussed on piperacillin hypersensitive patients with CF to begin to describe the different molecular events and components of the drug-specific humoral immune response.

Thus, the main objectives of this thesis were to:

1. Modify existing and develop new methods to detect drug-specific B-cell antibody secretion in piperacillin tolerant and hypersensitive patients with CF.
2. Generate and characterize drug-protein adducts and detect anti-drug antibodies in piperacillin hypersensitive patients with CF.
3. Define the role(s) of these anti-drug antibodies on T cell proliferation during immune reactions in piperacillin hypersensitive patients.
4. Develop methods for long term culture of B-cell lines and generation of anti-drug antibodies.

CHAPTER 2

MATERIALS AND METHODS

Table of Contents

Figures

Tables

2.0 Introduction

This chapter aims to showcase the various methods employed in the search for the underlying mechanisms explaining the role of B-cells in the pathogenesis of drug hypersensitivity disorders specifically those due to β-lactam antibiotics. This chapter has been divided into three general sections to enhance the clarity of the methods used. These are broadly the cell culture methods which includes cell isolation and culture, functional assays which covers the detection of secretory products from cells which helps to define to a certain extent their form and function, and lastly the proteomic studies which includes the identification of drug-protein interactions.

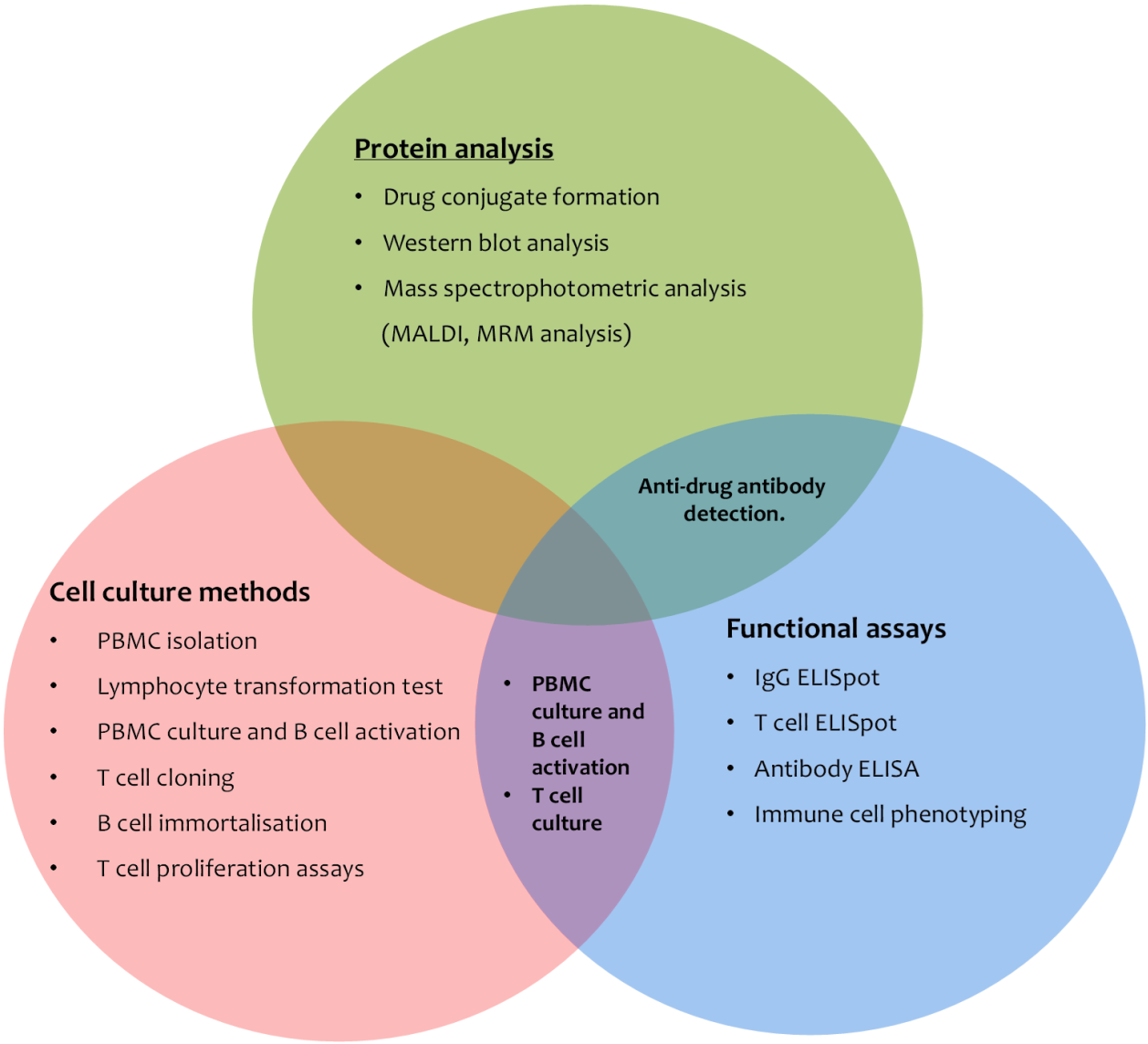


Figure 2.1 Figure showing the general assays employed. Some assays were multifunctional in the sense that they helped link two aspects of the study.

2.1 Cell culture methods

The cell culture methods used form the basic foundation of this thesis and were based on methods that had been previously tested in various research groups and others modified to suit the aims of this thesis.

2.1.1 Media for cell culture, cell separation and flow cytometry analysis

The ingredients used in the preparation of the various culture media, and preparations for cell separation and flow cytometry analysis are represented in the table below. Other materials employed in this thesis are listed in appendices I – III.

Table 2.1 Media for cell culture, cell separation and flow cytometry analysis. Table showing the different preparations used in the culture, separation and phenotypic analysis of cells. The constituents of these media and sources are also listed.

Preparation	Contents/source
F1	1. RPMI 1640 (Sigma R0883) 2. 10% pooled FBS (Gibco 10270) 3. HEPES buffer 25mM (Sigma H0887) 4. L-glutamine 2mM (Sigma G7513) 5. Streptomycin 100 mg/ml 6. Penicillin 100 U/ml (Sigma P0781)
R9	1. RPMI 1640 (Sigma R0883) 2. 10% pooled human blood type AB serum (Innovative Research HP1022) 3. HEPES buffer 25mM (Sigma H0887) 4. L-glutamine 2mM (Sigma G7513) 5. Transferring 25 mg/ml (Sigma T0665) 6. Streptomycin 100 mg/ml 7. Penicillin 100 U/ml (Sigma P0781)
R10	1. RPMI 1640 (Sigma R0883) 2. 10% pooled FBS (Gibco 10270) 3. HEPES buffer 25mM (Sigma H0887) 4. L-glutamine 2mM (Sigma G7513) 5. Streptomycin 100 mg/ml 6. Penicillin 100 U/ml (Sigma P0781) 7. 2-mercaptoethanol (Concentrated 2 - ME was diluted to 50 mM in PBS and added to media to attain a final concentration of 50 μm)
FACS buffer	1. Hanks balanced salt solution (HBSS) (500mL) 2. Fetal bovine serum (50μL) 3. Sodium azide (100mg)
10x MACS buffer	1. Hanks balanced salt solution (HBSS) (47.5 ml) 2. Ethylenediaminetetraacetic acid (EDTA) (2 ml) 3. Bovine serum albumin (2.5 g)

2.1.2 Peripheral blood mononuclear cells (PBMC) isolation from blood using density centrifugation

Twenty millilitres (20 ml) of lymphoprep was introduced into a 50 ml tube, and an equal volume of fresh venous heparinised blood collected from healthy volunteers or cystic fibrosis patients was layered onto the lymphoprep to form two distinct layers. The tubes were then centrifuged at 2000 rpm, for 25 minutes, with the absence of brake (brake = 0). After the spin cycle the tubes showed three distinct phases, with a thin layer of cells nestling between the top two layers (plasma and lymphoprep). Using a pasteur pipette the thin layer of cells was carefully removed from the tube and transferred into a sterile 50 ml tube. The tubes were filled up with hanks balanced salt solution (HBSS) and centrifuged at 1800 rpm, for 15 minutes, brake = 3. This was performed to facilitate washing of the cells. After the spin cycle the supernatant was discarded and the cells re-suspended in a small quantity of HBSS. At this point it was beneficial to contain all the cells in as few tubes as possible, thus necessitating pooling together cell pellets from the same donor. The tube containing the pooled cells was then filled with HBSS and centrifuged at 1500 rpm, for 10 minutes and brake = 3. After this spin the supernatant was discarded and the cells resuspended in a fixed volume of HBSS or culture medium. Trypan blue was added to an aliquot of cells prior to counting using a haemocytometer. Cells were then seeded in culture plates or froze by cryopreservation in foetal bovine serum + 10% DMSO at -150^{o}c.

2.1.3 Lymphocyte transformation test

PBMCs were isolated and counted as described earlier. The PBMCs were then cultured in triplicate using 96-well U-bottom plates at a cell density of 1.5 x 10^5 cells/well in 200 µl per culture condition (culture medium, titrated drug, and tetanus toxoid 5 µg/ml; positive control). The plates were then labelled, wrapped in foil and stored at 37^0C, 5% CO2, for five days. 0.5 µCi of thymidine [^{3}H] was added to each well for the final 16 hr of incubation. At the end of the incubation period the plates were harvested on filter mats using a microplate harvester (TOMTEC harvester 96 MACH III M), and the extent of thymidine incorporation measured as counts per minute (cpm) using a beta counter (Perkin ELMER microbeta Trilux). Analysis of cell proliferation was denoted by the stimulation index (SI), of which any SI value $\geq$ 2 was accepted as a positive result (Nyfeler and Pichler 1997, Pichler and Tilch 2004).

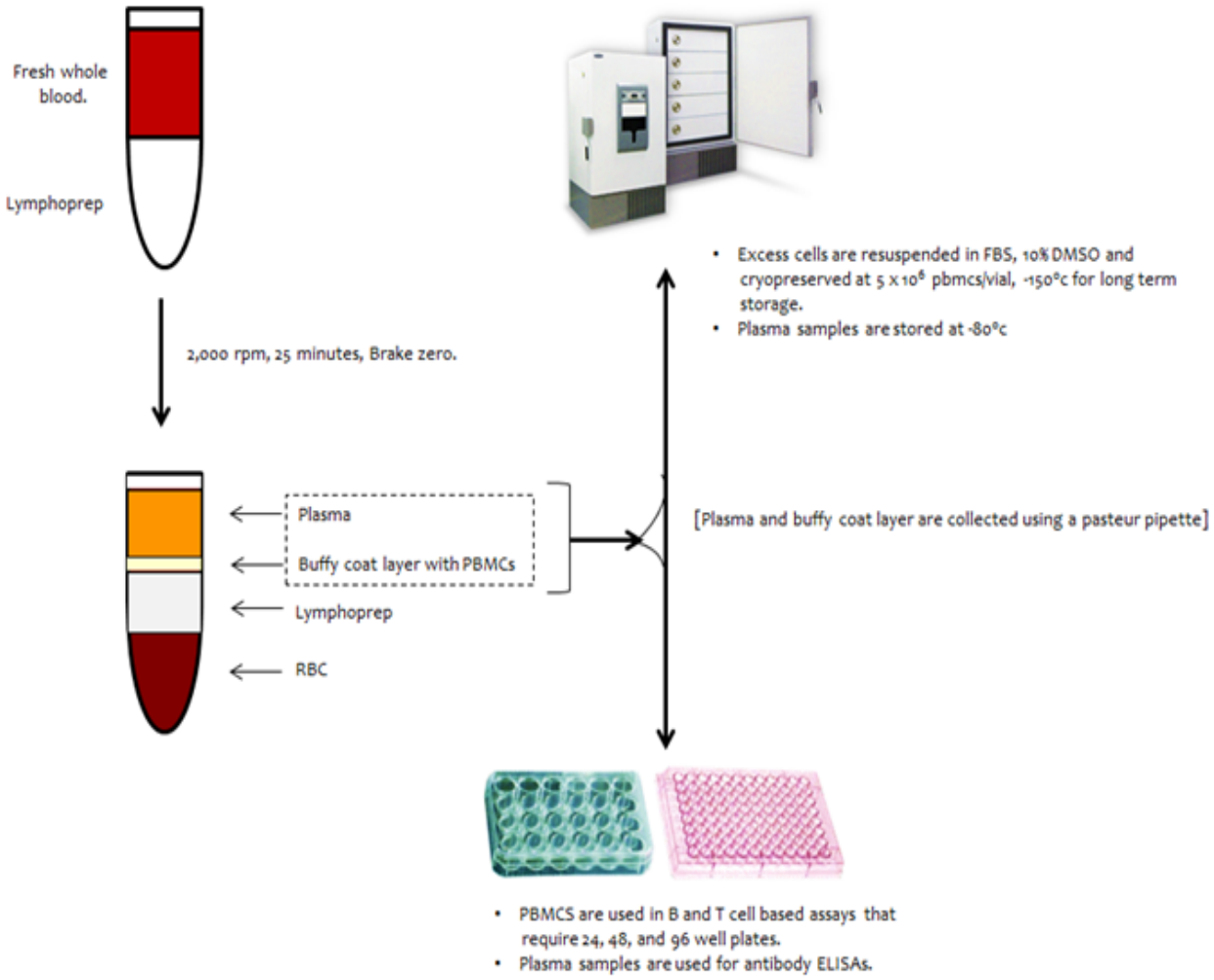


Figure 2.2. Schematic representation of the PBMC isolation and their various downstream applications.

2.1.4 PBMC culture and B-cell culture and activation studies

Isolated PBMCs were re-suspended at 1 x 10^6 PBMC/ml in 1ml R10 (see constituents in section 2.2). Various culture conditions were prepared including 1.5 µg/ml Cpg DNA mitogen stimulation medium and a 2 mM solution of piperacillin stimulation medium, in R10 medium sufficient for 1 well (1 ml/well) per treatment condition. Using sterile techniques, 1 x 10^6 PBMCs were added to each well containing R10 culture medium alone, 2 mM piperacillin, and Cpg-DNA in a 24-well plate. These plates were then placed in 5% CO_2 at 37°C for 5 days. On the fifth day, plates were harvested with PBMCs and cell supernatants collected for ELISA and ELISpot analysis (Fig 2.2).

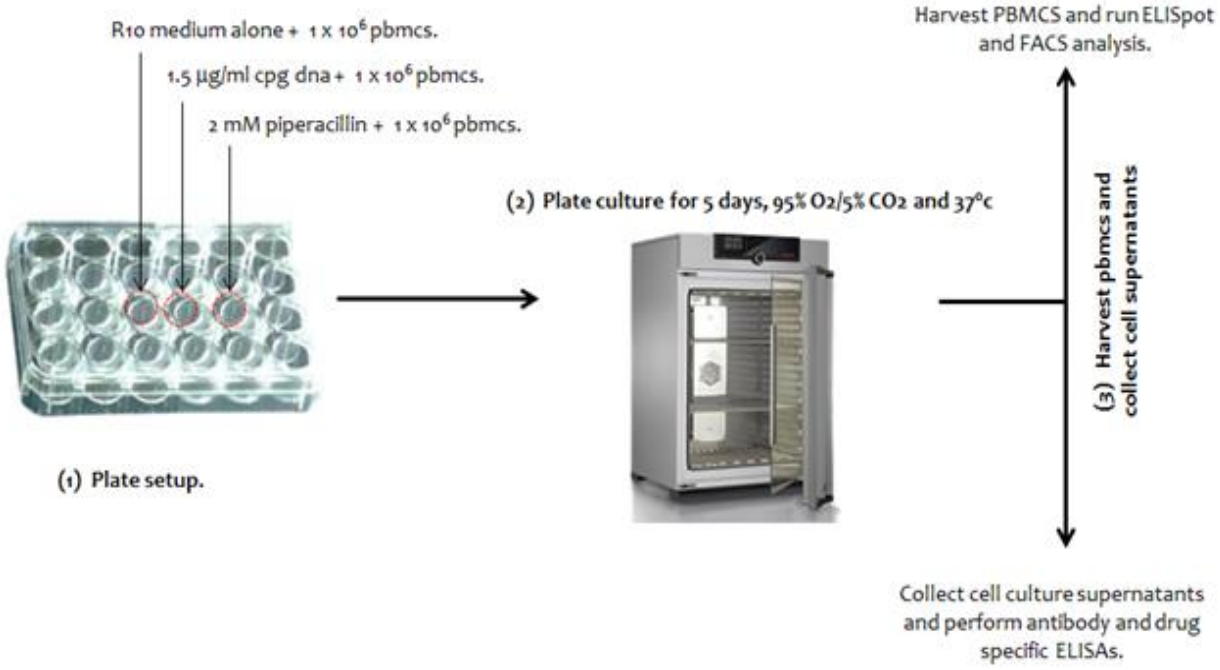


Figure 2.3 Figure showing schematic diagram of PBMC culture and B cell activation as a starting point for the functional assessment of lymphocytes.

2.1.5 CD19 positive PBMC separation

Isolated PBMCs were centrifuged and re-suspended in 80 µl MACS buffer per 10^7 cells, to which 20 µl of vortexed CD19 micro beads were added per 10^7 cells. Mixing was facilitated by gentle agitation and the mixture incubated for 15 minutes at 4°C. Note that for samples with less than 10^7 cells 80 µl/20 µl volume ratios of Macs buffer and CD19 micro beads was used. After incubation Macs buffer was added per 10^7 cells and tubes were centrifuged at 1500 rpm for 8 minutes at 4°C. The supernatant was pipetted off completely and cells re-suspended at 10^8 cells per 500 µl Macs buffer. An MS column (Miltenyi Biotech GmbH Germany) was placed on a magnet and washed with 500 µl Macs buffer. Cells were then added to the column in 500 µl aliquots. The tube was rinsed once with 500 µl Macs buffer. The column was then washed three times with 500 µl Macs buffer, the buffer being added separately once the reservoir was empty. The flow through containing negative cells was collected in 5 ml bijou tubes. The column was removed from the magnet and placed over a collection tube. 1 ml Macs buffer was added to the column and selected cells harvested by immediately applying the plunger firmly to the column. The number of CD19 positive B cells was counted using trypan blue exclusion with the aid of a haemocytometer.

2.1.6 Generation of IgG secreting B-cell lines

Long lived IgG secreting B cells were generated using methods modified from that employed by Lanzavecchia et al. (Lanzavecchia, Corti et al. 2007). Isolated PBMCs from patients bearing the CD19 phenotype were utilised for this assay. They were obtained using previously described CD19 positive separation techniques. Thirty to a hundred (30 – 100) CD19+ B cells per well were cultured in U-bottomed 96-well plates

in the presence of 1 x 10^5 irradiated PBMCs per well which are employed as feeder cells. 1 µg/ml CpG-dna, 30% w/v Epstein barr virus (EBV), and 1 mM piperacillin were also added. This culture was kept at 37°C, and 5% CO_2 for 14 days. Care was taken to feed B cells with F1 medium supplemented with 0.01 mM sodium pyruvate twice weekly. After two weeks the volume of each well was reduced to 100 µl and 1 µg/ml Cpg-dna and 50 U/ml IL-2 added to give a total volume of 200 µl/well. Cells were kept under earlier prescribed culture conditions. Growing wells were then transferred to 48-well culture plates to enhance cell proliferation and expansion. After an additional two weeks, culture supernatants were tested for IgG secretion using a total IgG enzyme linked immunosorbent assay (ELISA) and cells harvested to test for IgG secretion using an enzyme linked immunospot (ELISpot) assay (see figure 2.3). IgG secreting lines were then assayed for the presence of anti-drug specific IgG.

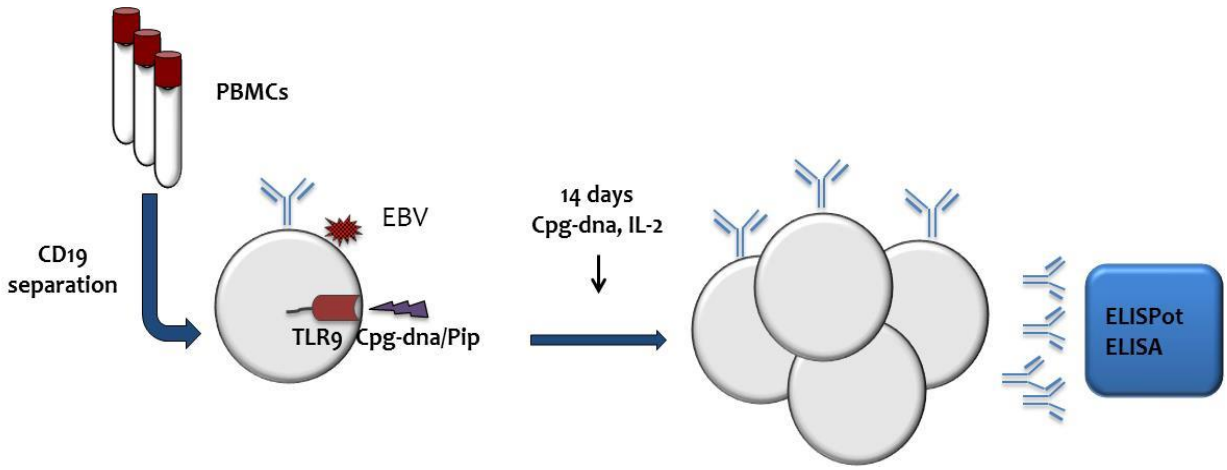


Figure 2.4. TLR9 activation and viral induced immortalization of B cells for the generation of IgG secreting B cell lines.

2.1.7 Generation of drug-specific T- Cell clones

2.1.7.1 Drug Dilutions

Piperacillin was dissolved in R9 medium to create a stock solution of 4 mM. PBMCs were counted and diluted to 3 x 10^6 cells/ml**.** 330 µl cells and 330 µl piperacillin were seeded onto a 48 well plate to give a final drug concentration of 2 mM with 2 wells per concentration. Cells were cultured for 14 days under prescribed culture conditions and were referred to as 'bulk cultures'. On the 6^{th} and 9^{th} days in culture 330µl IL-2 in R9 medium was added.

2.1.7.2 Serial dilution

On day 14 all 4 wells were mixed, cells counted, and the total bulk culture diluted serially. The cells were diluted to a stock solution of 1 x 10^4 cells/ml. Three cell concentrations were then prepared each utilising 9 µl, 30 µl, and 90 µl of the 1 x 10^4 stock in a 35 ml serial dilution cocktail. The serial dilution cocktail consisted of irradiated PBMC from an allogenic donor [5 x 10^4 PBMC/well, irradiated for 15 minutes], PHA [5 µg/ml], IL-2 [2.5 µl/ml of the 10^5 stock solutions] was added and the required amount of 1 x 10^4/ml stock cells for each condition into 35 ml R9 medium. 96 well U-bottomed culture plates were used and the final T-cell number was 0.3 cells/well, 1 cell/well and 3 cells/well after seeding with 100 µl of the individual cocktails. Cells were cultured for 14 days. Medium was supplemented on day 5 and then every 2 days with 25 µl IL-2 in R9 medium (from a 2.5 µl/ml solution of the 10^5 unit stock solution).

On the 14^{th} day the volume of the wells was reduced to 80 µl and a 're-stimulation' cocktail which consisted of irradiated cells from an allogenic donor (5 x 10^4 PBMCs/well,

irradiated for 15 minutes), PHA (10 µg/ml), IL-2 (7 µl/ml of the 10^5 stock solution) was added. Feeding was carried out every 2 days with the addition of 25 µl IL-2 in R9 medium (from a 2.5 µl/ml solution of the 10^5 stock solution). Well growing wells distinguished by the formation of a solid pellet and a colour change of the cell culture medium from pink to yellow, were transferred and plated out in 2 wells in a new 96 U bottom well plate. Wells were split from 2 to 4 when further change of the 2 wells to yellow was observed and feeding was continued every 2 days.

2.1.7.3 Testing for T-cell clone drug specificity

Testing for drug specificity was carried out on clones that had proliferated sufficiently to be expanded to at least 4 wells on 96 well U-bottomed plates. The volume in each well of the 96 U bottomed plates was reduced to 100µl. Cells from 2 wells were then mixed and transferred to 4 wells (50 µl/well) in a new 96 well U-bottomed plate. 1 x 10^4 autologous irradiated EBV transformed B cells (20 minutes in the presence of ^{123} [Cs]) were added in 50 µl R9 medium. 100 µl of piperacillin (4 mM) was added to 2 of the 4 wells and R9 medium alone to the other 2 wells. Plates were incubated for 48 hr, 37^{0}c, 5% CO_2 and 0.5 µCi/well ^{3}H thymidine added for the last 16 hr. The proliferative response was analysed using a beta counter and clones that achieved S I values $\geq$ 2 when compared to R9 were considered as drug specific. Clones which did not exhibit drug specificity were discarded.

2.1.7.4 Re-stimulation of drug-responsive T-cells

Re-stimulation cocktail was prepared by mixing irradiated cells from any donor (5 x 10^5 cells/well, irradiated for 15 minutes), PHA (10 µl/ml), and IL-2 (7 µl/ml of 10^5 stock). The two remaining 96U wells of each drug specific clone were mixed and transferred into

sterile FACS tubes. Each tube was then made up to 1 ml with R9 and centrifuged at 1500 rpm for 5 minutes. Clones were then re-suspended in 330 µl R9, plated onto a 48 well plate and 330 µl of the re-stimulation cocktail was then added to each well. The following day and every 2 days thereafter 330 µl R9 with IL-2 (2.5 µl/ml) was added.

2.1.8 Epstein Barr virus (EBV) induced immortalisation of B – Cells

B95-8 cells are an extensively used cell line obtained from the marmoset monkey which secretes the B95-8 strain of Epstein-Barr virus (EBV). The B95-8 cell line was initially immortalized by infection with virus from the 883L cell line, which was acquired through the culture of lymphocytes from an elderly person with transfusion-induced infectious mononucleosis (Miller, Shope et al. 1972, Skare, Edson et al. 1982).

B95-8 cells were centrifuged and 8 ml of supernatant was removed. 5 ml of the supernatant was filtered (using a 0.2 µm filter) onto 5 x 10^6 PBMCs followed by the addition of 1 µg/ml cyclosporin A (CSA). Cells were incubated at 37°c, 5% CO_2. Post incubation the cells were centrifuged at 1500 rpm for 5 minutes and the supernatant discarded. PBMCs were then re-suspended in 2 ml F1 medium + CSA (1 µg/ml) from which 1 ml, 0.5 ml, and 2 x 0.25 ml volumes respectively were plated into 4 wells on a 24 well plate. F1 medium was then added to create a total volume of 1 ml in each well. The cells were then fed with F1 + CSA twice a week for 3 weeks. From this point forward CSA was removed from the culture medium. Splitting was performed when the medium in the wells started to turn yellow. Feeding was continued twice a week with F1 medium and cells transferred into 25 ml flasks and later into 60 ml flasks. Some cells were frozen at -

150°C or in liquid nitrogen for long term storage using methods described in section 2.1. Lines were maintained at 37°C, 5% CO_2 using a twice weekly feeding cycle.

2.1.9 T- Cell proliferation assay

Drug-specific T-cell clones (50 X 10^4 cells/well; 100 µl) were seeded in duplicate in a U-bottomed 96 well plate, with varying drug concentrations in medium appropriate for the culture of T cells. To these wells ^{123}Cs irradiated autologous EBV transformed B cells (1.0 x 10^4 EBVs/well) were added, to achieve a total well volume of 200 µl after which the plates were incubated at 37°C, 5% CO_2 for 48 hr. Cells were pulsed with [^3H] thymidine for the last 16 hr of culture, and plates were subsequently harvested and incorporated radioactivity counted with a beta counter. Proliferation was calculated as the SI as described previously, and SI values ≥ 2 were considered positive.

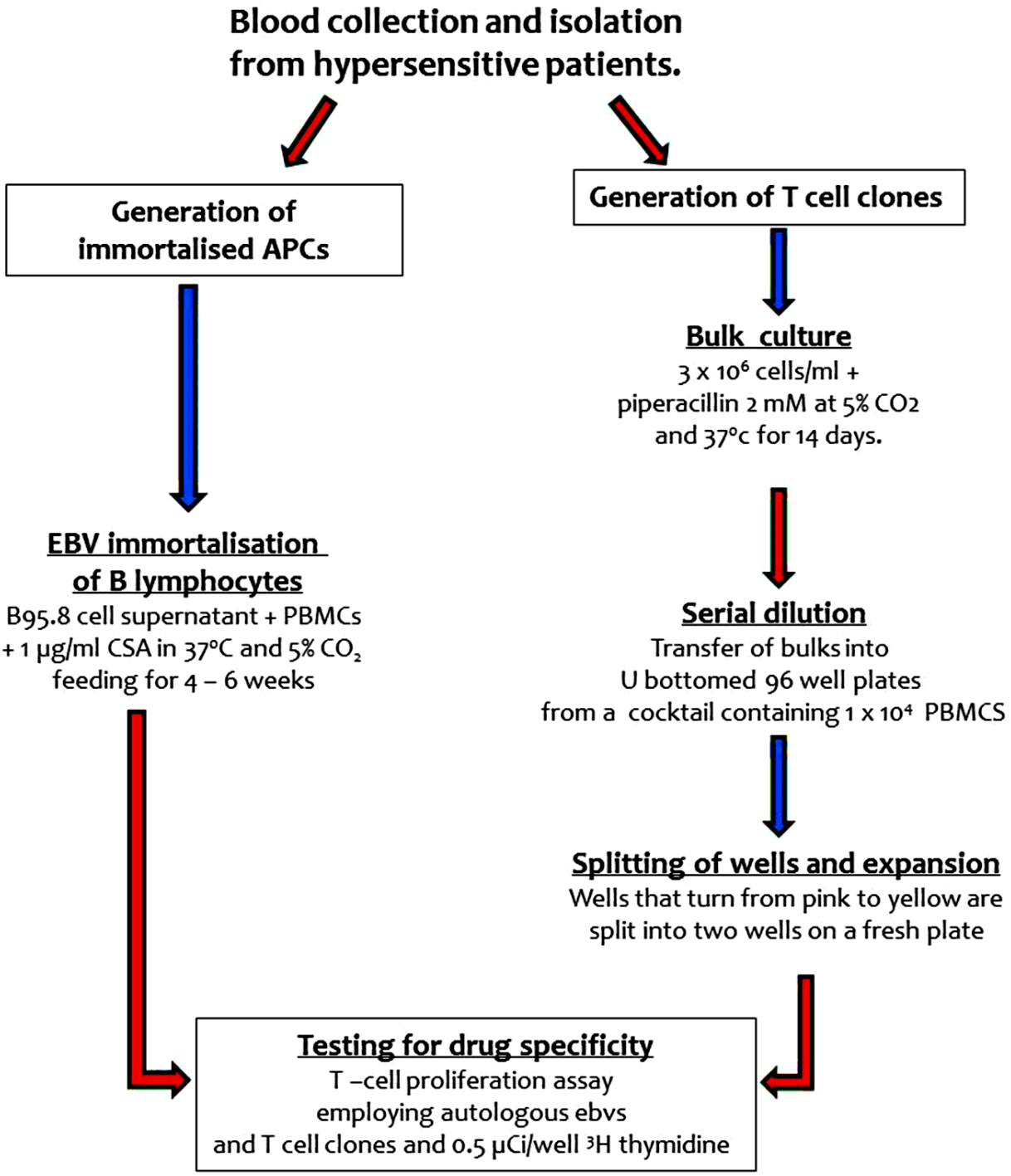


Figure 2.5 Schematic diagram of the generation of autologous B cell lines, T cell clones and analysis of antigen-specific T cell responses.

2.2 Functional assays

This section covers assays carried out to define the forms and function of cells and their secretory products and endogenous marker expression.

2.2.1 Enzyme linked immunospot (ELISpot) assay

The *in-vitro* cell culture of PBMCs or cell lines following incubation with antigens or mitogens leads to the production of antibodies and cytokines, and the number of cells expressing these molecules may be detected using an enzyme linked immunospot (ELISpot) assay which has been documented previously (Czerkinsky, Nilsson et al. 1983, Crotty, Aubert et al. 2004, Cao, Gordic et al. 2010). The ELISpot assay is presumed by some to be the gold standard for the enumeration of antigen-specific B-cells (Bonsignori and Moody 2012, Jahnmatz, Kesa et al. 2013). ELISpots were conducted using drug stimulated PBMCs and B-cell lines to detect the secretion of immunoglobulin G (IgG), but also PBMCs and drug-specific lines and clones to detect the secretion of interleukin-5 (IL-5), interleukin-13 (IL-13), interferon gamma (IFN-γ), and granzyme B (GB) and interleukin-22 (IL-22).

2.2.1.1 Sterile Preparation of ELISpot plate

This procedure was carried out in a class II microbiological safety cabinet. The coating antibodies with specificity for IgG or various cytokines were diluted to their required concentration in sterile PBS, pH 7.4. The polyvinyl difluoride (PVDF) membrane was pre-wet by the addition of 100 µl of 35% ethanol per well for 1 minute, after which the membranes were washed 5 times with sterile Hanks balanced salt solution (HBSS), using

200 µl/well each time. 100 µl/well of the required coating antibody solution was added and the plate incubated overnight at 4°C.

2.2.1.2 Incubation of PBMCs for detection of IgG antibody secreting B lymphocytes

PBMCs harvested from the 5 day PBMC culture described in section 2.3 were suspended in 1 ml of R10 medium and cell numbers for each treatment group determined using a haemocytometer. Excess antibody was removed from the ELISpot plate via washing as described above. 200 µl/well of R10 medium, the same as that used for the cell suspensions, was then added onto the plate and incubated for 30 minutes at room temperature. After this incubation, the medium was removed and 5 x 10^4 cells from PBMCS earlier pulsed with drug, Cpg-dna or medium control were added into separate wells (3 per treatment group) with each well containing a maximum volume of 200 µl. The plate was then wrapped in foil and kept at 37°C, 5% CO_2 for 24 hr.

2.2.1.3 Incubation of PBMCs for detection of cytokine secreting T-lymphocytes

The excess antibody was removed and the plate washed as described above. 200 µl/well of R9 medium, the same as that used for the cell suspensions was then added onto the plate and incubated for 30 minutes at room temperature. The medium was then removed and 100 µl/well of the stimuli which in this case was piperacillin (2 mM) and PHA (5 µg/ml) was added followed by 100 µl/well of the cell suspension containing 5 x 10^5 PBMCs. The ELISpot plate was incubated at 37°C, 5% CO_2 and incubated for 24 hr. Care was taken not to move the plate during this time and the plate was also wrapped in foil to prevent evaporation.

2.2.1.4 Incubation of drug-specific T-cell clones for detection of T-cell cytokines

The excess antibody was removed and the plate washed as described above. 200 µl/well of R9 medium, the same as that used for the cell suspensions was then added to the plate and incubated for 30 minutes at room temperature. The medium was then removed, and 100 µl/well of the stimuli added to achieve a final drug concentration of piperacillin 2 mM, PHA (5 µg/ml) and medium control. This was followed by the addition of 50 µl/well of the cell suspension containing 1.5 x 10^5 T-cell clones PBMCs, and 50 µl/well of a cell suspension containing 5 x 10^4 autologous EBV-transformed B cells. The ELISpot plate was incubated at 37°C, 5% CO_2 and incubated for 48 hr. Care was taken not to move the plate during this time and the plate was also wrapped in foil to prevent evaporation.

2.2.1.5 ELISpot membrane development and spot detection

The cells were removed from the plate and wells washed 5 times with 200 µl/well HBSS. Detection antibodies for the respective IgG and T-cell cytokines were diluted to their preferred concentrations in PBS containing 0.5% fetal calf serum (PBS-0.5% FCS) and 100 µl added to each well. The plates were then incubated for 2 hr at room temperature. After incubation the plates were washed 5 times as previously described and 100 µl Streptavidin-ALP (1:1000) in PBS-0.5% FCS added to each well. After incubation for 1 hr at room temperature the plates were washed and 100 µl/well of BCIP/NBT substrate solution was added and the plate observed until distinct spots emerged. Colour development was stopped by washing extensively under flowing tap water. Plates were then left to dry, after which the spots were counted on an ELISpot plate reader (ELISpot reader ELR06;AID Advanced Imaging Devices, GmbH Germany).

2.2.2 Flow cytometry

2.2.2.1 CD3, CD19 and CD27 phenotype expression in activated PBMCS

Phenotypical identification of CD3, CD19, and CD27 expressing lymphocytes was carried out using flow cytometry. Cells taken from PBMC antigen or mitogen cultures as described in section 2.11.2 were split between fluorescent activated cell sorting (FACS) tubes. Two conditions one stained with appropriate antibody and the other stained with isotype control were then set up for each treatment group. PBMCs were then washed by centrifuging the FACS tubes at 1500rpm, 5minutes, and brake = 3 after which the supernatant was discarded carefully. 200µl of FACS buffer was then added to each tube. Approximately 3 µl of each of CD3, CD19 and CD27 fluorochrome antibodies, depending on the phenotype to be determined was added in the dark to only the stained sample tubes. The samples in both tubes were then incubated in the fridge for 20 minutes at 4°C, and covered in foil. After 20 minutes, 1 ml FACS buffer was added and the cells were washed. The samples were then either re-suspended in 200 µl of FACS buffer for same day analysis, or 200 µl 4% paraformaldehyde for next day analysis. The fixing process has been shown to be suitable for up to 30 hr storage at 4°C. The samples in FACS buffer can be stored at 4°C for a few hr covered with foil until required.

2.2.2.2 CD4, CD8 phenotypical characterization of drug-specific T-cell clones

The phenotype expressed by drug specific T cell clones was also carried out using flow cytometry. T cell clones from expanded 48 well plates and confirmed to be drug specific were transferred into two FACS tubes per clone, ensuring that there was a maximum of 5 x 10^5 PBMCs in each tube. Two conditions, one stained and the other unstained were

then set up for each treatment group. T cell clones were then washed as described above and suspended in 200µl of FACS buffer was then added to each tube. 3 µl of CD4 and CD8 fluorochrome antibodies, were added in the dark to stained sample tubes alone. The samples were incubated in the fridge for 20 minutes at 4°C covered in foil. After 20 minutes, 1 ml FACS buffer was added and a wash centrifuge cycle performed. The supernatant was then discarded. The samples were then either re-suspended in 200µl of FACS buffer for same day analysis, or 200µl 4% paraformaldehyde (PFA) for next day analysis.

2.2.3 Enzyme Linked Immunosorbent Assay (ELISA)

The identification of drug-specific antibodies was attempted using a modified protein for antigen (drug molecule). This is made possible by employing the use of drug-protein adducts prepared by the incubation of the drug with a model protein and then precipitation with an appropriate alcoholic precipitant (Ariza, Garzon et al. 2012) ; See section 2.3.1 for the precipitation of adducts. Flat-bottomed 96 well microplates were pre-coated with modified and unmodified protein-drug adducts, and the difference in the levels of antibodies detected between both conditions after plasma sample addition referred to as the amount of anti-drug antibody specific to the particular drug of interest. This may be confirmed by the pre-incubation of the plasma samples with known quantities of an inhibitor before addition to the microplate to see if the expression of circulating anti-drug antibodies will be nullified (Coleman, Yeung et al. 1986, Christie, Coleman et al. 1988, Clarke, Neftel et al. 1991, Zhang, Wang et al. 1996).

2.2.3.1 ELISA for the detection of total IgG levels in volunteer and patient plasma samples

The method employed was the same as described using the commercial IgG detection kit (Mabtech). Microtitre plates were coated overnight at 4°C with 100 µl of a 1:100 solution of anti-IgG coating antibody (0.05 M phosphate buffer, pH7.2; 100 µl/well). After overnight incubation the plates were washed 3 times with 200 µl/well 0.15 M PBS containing 0.05% Tween 20 (PBS-Tween, pH 7.2) and tapped dry. Plates were blocked with 100 µl of a non-specific protein (BSA) for 30 minutes, washed as previously described and then incubated with 100 µl of volunteer or patient serum serially diluted ten-fold and then incubated for 1 hr at room temperature. Goat anti-human IgG was diluted to 1/2000 in PBS/tween and 100 µl was added to the plates. They were then left to incubate at room temperature for one hr. Plates were washed three times and 100µl of horseradish peroxidase-labeled rabbit anti-goat IgG (1/5000 in PBS-Tween) was added to each well. After a 1 hr incubation at room temperature 100 µl of TMB substrate was added to each well and the plates left for 20 minutes in the dark. The enzyme-substrate reaction was then terminated by addition of 100 µl of 0.18 M sulphuric acid. Absorbance values were read at 490 nm using dual wavelength automated plate reader (Dynatech MR600) with the reference wavelength set at 630 nm.

2.2.3.2 ELISA for the detection of antidrug antibody levels in tolerant and hypersensitive patient plasma samples

Microtitre plates were coated overnight at 4°C with 100 µl of a 20 µg/ml concentrated solution of drug adducts (modified and unmodified HSA or BSA conjugates in 0.05 M phosphate buffer, pH7.2; 100 µl/well). After overnight incubation the plates were washed

three times with 200 µl/well of a 0.15 M PBS containing 0.05% Tween 20 (PBS-Tween, pH 7.2) and tapped dry. Plates were blocked with 100 µl of a non-specific protein for 30 minutes, washed as previously described and then incubated with 100 µl of volunteer or patient serum serially diluted ten-fold and incubated at room temperature for 1 hr. Goat anti-human IgG was diluted to 1/2000 in PBS/tween and 100 µl was added to the plates. They were then left to incubate at room temperature for one hr. Plates were washed three times and 100µl of horse radish peroxidase-labelled rabbit anti-goat IgG (1/5000 in PBS-Tween) was added to each well. After a 1 hr incubation at room temperature 100 µl of TMB substrate was added to each well and the plates left for 20 minutes in the dark. The enzyme-substrate reaction was then terminated by addition of 100 µl of 0.18 M sulphuric acid. Absorbance values were read at 490 nm using dual wavelength automated plate reader (Dynatech MR600) with the reference wavelength set at 630 nm.

2.2.3.3 ELISA for the detection and quantification of IgG subclasses and drug specific antibody subclasses in tolerant and hypersensitive patient plasma samples

Microtitre plates were coated overnight at 4°C with 100 µl of a 1:100 concentrated solution of anti-IgG coating antibody (0.05 M phosphate buffer, pH7.2; 100 µl/well). After the overnight incubation the plates were washed three times with 200 µl/well 0.15 M PBS saline containing 0.05% Tween 20 (PBS-Tween, pH 7.2) and tapped dry. Plates were blocked with 100 µl of a non-specific protein (BSA) for 30 minutes, washed as previously described and then incubated with 100 µl of volunteer or patient serum serially diluted ten-fold and then incubated for 1 hr at room temperature. Horse radish peroxidase-labelled mouse anti human IgG1, IgG2, IgG3 and IgG4 (Invitrogen) and NOR-01 human

serum standard for IgG sub-classes (Nordic immunology) were diluted to their appropriate concentrations (IgG1- 1:1000; IgG2-1:10,000;IgG3-1:10,000 and IgG4-1:10,000) in PBS (pH 7.4) and 100 µl was added to the plates. They were then incubated at room temperature for one hr. Plates were washed 3 times and 100 µl of streptavidin diluted 1:1000 for IgG1 and 1:10,000 for IgG2, IgG3 and IgG4 were added to each well and left to incubate for 1 hr at room temperature. 100 µl of TMB substrate was added to each well and left for 20 minutes in the dark. The enzyme-substrate reaction was terminated by addition of 100 µl of 0.18 M sulphuric acid. Absorbance values were read at 490 nm by dual wave length automated plate reader (Dynatech MR600) with the reference wavelength set at 630 nm. IgG antibody titers were calculated as previously described.

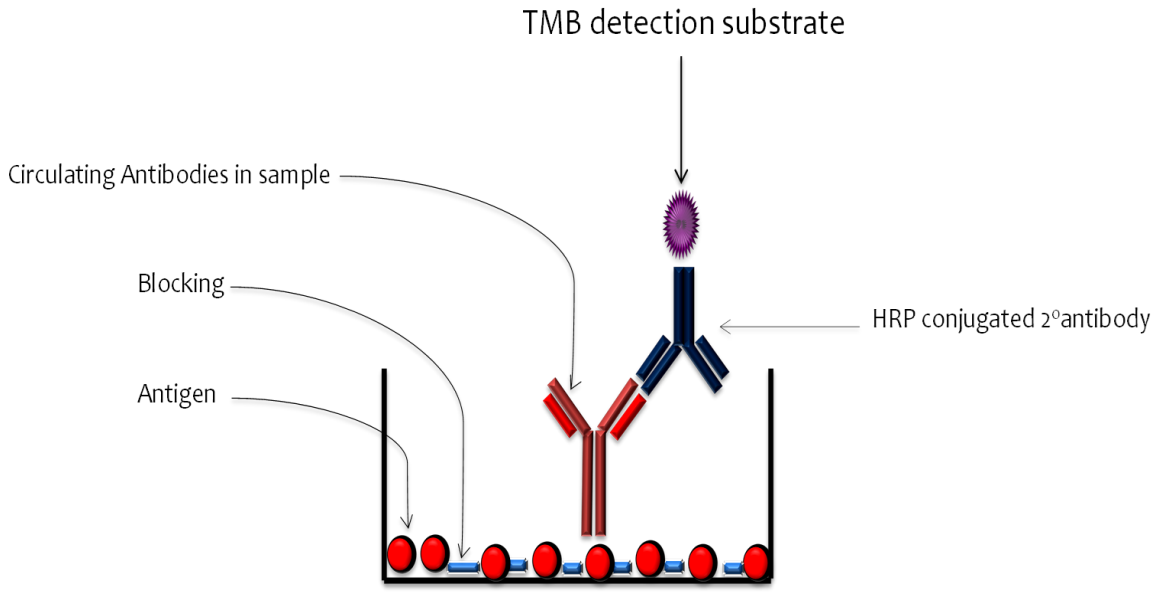


Figure 2.6 Diagram of the enzyme linked immunosorbent assay (ELISA). The ELISA was employed in the detection of circulating antibodies in hypersensitive patient plasma and cell culture supernatants.

2.2.3.4 ELISA for the detection of total IgG levels in patient PBMC culture supernatants

The method employed was the same as described using the commercial IgG detection kit (Mabtech). Microtitre plates were coated overnight at 4°C with 100 µl of a 1:100 solution of anti-IgG coating antibody (0.05 M phosphate buffer, pH7.2; 100 µl/well). After overnight incubation the plates were washed 3 times with 200 µl/well 0.15 M PBS containing 0.05% Tween 20 (PBS-Tween, pH 7.2) and tapped dry. Plates were blocked with 100 µl of an non-specific protein (BSA) for 30 minutes, washed as previously described and then incubated with 100 µl of volunteer or patient 5 day pbmc culture (culture medium, CpG-dna and piperacillin 2 mM) supernatants serially diluted ten-fold and then incubated for 1 hr at room temperature. Goat anti-human IgG was diluted to 1/2000 in PBS/tween and 100 µl was added to the plates. They were then left to incubate at room temperature for one hr. Plates were washed three times and 100µl of horseradish peroxidase-labeled rabbit anti-goat IgG (1/5000 in PBS-Tween) was added to each well. After a 1 hr incubation at room temperature 100 µl of TMB substrate was added to each well and the plates left for 20 minutes in the dark. The enzyme-substrate reaction was then terminated by addition of 100 µl of 0.18 M sulphuric acid. Absorbance values were read at 490 nm using dual wavelength automated plate reader (Dynatech MR600) with the reference wavelength set at 630 nm.

2.3 Protein analysis

In this section we illustrate the methods employed in the preparation and characterization of synthetic β-lactam conjugates generated in the laboratory.

2.3.1 Preparation of drug adducts

Drug modified protein adducts were generated and employed in functional assays and LC-MS characterization.

2.3.1.1 Methanol precipitation of β-lactam – protein adducts

i. Drug-protein incubations. To prepare drug adducts amoxicillin, flucloxacillin, penicillin V and piperacillin β-lactams were incubated with HSA and BSA at different protein-drug molar ratios. A 1 Molar (66 mg/ml) stock of HSA and BSA was prepared and used in all experiments. Drugs and proteins were prepared in PBS. Twenty four tubes were prepared for each condition. Protein and drugs were combined in the ratio HSA control (unmodified), drug-protein 20:1, drug-protein 50:1, and drug-protein 100:1 each with a total volume of 200 µl and incubated at 37°C for 24 - 96 hr.

ii. Methanol precipitation. 1800 µl of ice-cold methanol was added to the 200 µl sample and the tubes were maintained on ice. One microlitre of 10% trifluoroacetic acid (TFA) was added to samples that did not precipitate. The samples were then centrifuged at 17000g/14000rpm, 4°C for 15 minutes. Care was taken to pipette the methanol from the eppendorf tubes. 100 µl of the phosphate buffer was then added to each tube, which were vortexed and mixed with a pipette. The tubes were then made up to 1 ml with 900 µl methanol to initiate a second precipitation step. The sample was centrifuged as previously described. 50 µl of protein free Roswell park memorial institute (RPMI-1640) medium at pH 8 was added to each tube to dissolve the pellet. The eppendorf tubes were then centrifuged at 13,000 rpm for 2 minutes and the supernatants from all the tubes pooled. A Bradford assay was conducted to quantify the total protein content of each adduct.

2.3.1.2 Spin column collection of β-lactam – protein adducts

Drug conjugates of different hapten densities of 1:1, 5:1, 10:1, 20:1, 50:1, and 100:1 were prepared using this method which was necessary to eliminate the presence of ethanolic solvents in the final adduct, to limit potential toxicity following their use in cell cultures. A 132 mg/ml solution of BSA in phosphate buffer was prepared by dissolving 594 mg of BSA in 4.5 ml of phosphate buffer. 500 mM and 250 mM solutions of piperacillin were prepared in phosphate buffer, but only 500 mM of other β - lactam antibiotics were prepared. The protein and drug solutions were combined at the prescribed ratios and incubated in 24 well plates for 96 hr. The mixture was then transferred from the wells into Millipore amicon ultra-4 centrifuges spin columns equipped with a 10,000 KDa filter. 3 ml of phosphate buffer was added into each column to make the volume up to 4 ml. The tubes were centrifuged at 4°c, 14,000rpm/4,000g, for 30 minutes. After the centrifugation step, 500 µl of the flow through from each sample was collected and stored at -80°c for future LC-MS analysis. Additional flow through was discarded. Addition of 3 ml phosphate buffer to the protein pellet, centrifugation and collection of the flow through were repeated four more times. After the fifth spin a 100 µl pipette was used to extract and transfer the filter contents into an eppendorf tube. A Bradford assay was performed to quantify the protein in each conjugate formed and western blotting carried out to confirm adduct formation. Once adduct formation was confirmed they were then applied to functional assays and LC-MS analysis was conducted to characterise and measure the extent of modification and specific amino acid residues modified.

2.3.2 Gel electrophoresis for confirmation of adduct formation

2.3.2.1 Western blot analysis

The western blot analysis is an important technique which is commonly employed for the separation and consequent identification of proteins based on their differing molecular weights (Liu, Mahmood et al. 2014).

i. Sample preparation. 5 μg/ml of the sample was prepared in HBSS. The sample was then mixed in a 4:1 ratio with Laemmli buffer (20 μl sample + 5 μl of Laemmli buffer). Laemmli buffer consisted of a tracking dye (bromophenol blue), glycerol to increase the density hence allowing the samples to sink into the grooves and 2-mercaptoethanol which reduces disulphide bonds and disrupts protein cross-links.

Samples were then placed in a heating block for 10 minutes at 100°c to denature the ordered structure thus ensuring movement in an electric field. After heating, the samples were ready for addition to the gel.

ii. Sample separation. All instruments were sprayed and cleaned with 70% ethanol before coupling the casting block to ensure that there was no hardened acrylamide on the surfaces. A 10 % resolving gel (Protogel, Protogel resolving buffer, Water, tetramethylenediamine (TEMED), 10 % ammonium persulfate (APS) ;0.1 g in 1 ml) was made up and added into the gel compartments 1 ml at a time to just below the comb line. 1 ml H_2O was added to the top and the gel was left to set for 20-30 minutes. The stacking gel (Protogel, Protogel stacking buffer, Water, TEMED and 10 % APS (0.1 g in 1 ml) was then made up with TEMED and APS added right at the end. Water was then poured off and combs inserted. The stacking gel was then added 1 ml at a time and left to set for 20-30 minutes. Electrophoresis buffer was used to flush out the wells then 4 μl of the see

blue marker was added to the first lane. Sufficient amounts of the sample (with a 10 well comb 12 µl of sample was added to each well, while for a 15 well comb 10 µl was used) was then added to the other wells as required. The gel was run at 300v, 30 mA per gel, for 1 hr at 4°C to prevent the generation of heat.

iii. Transfer: Following separation the gels were removed and proteins transferred onto 8.5 cm x 6.5 cm nitrocellulose paper. A transfer cassette was placed into a tray with the white side at the bottom, the black side lifted and then components added in a specified order; sponge, filter paper (8.5 cm x 6.5 cm), nitrocellulose membrane (8.5 cm x 6.5 cm), gel, filter paper (8.5 cm x 6.5 cm), sponge and transfer buffer. Bubbles were smoothened out by rolling a pipette over the cassette. Transfer buffer was then poured into a transfer tank, and attached to a 4°C cooler. The cassette was closed and put into the transfer tank with the black side to the black probe. The top was put on and transfer carried out at 300v, 250 mA for 1 hr.

iv. Ponceau stain and addition of the secondary antibody. After the protein transfer distilled water was placed in two glass dishes and the nitrocellulose membrane rinsed. The ponceau stain was then added for 1 - 4 minutes after which it was washed off the membrane with distilled water to visualise the protein. After visualization the membrane was washed in **TST** to remove the stain (TST: 8.76 g NaCl, 1.21 g Tris, 1 ml Tween in 1 litre of water). The nitrocellulose membrane was then kept in a plastic petri dish and approximately 20 ml of blocking buffer was added to the petri dish and rocked slowly at room temperature for 2 hr. The blot was then immersed in mouse anti-penicillin antibody (Sigma) diluted to 1:20,000 in 2.5 % blocking buffer (2.5 g milk in 100ml TST) or rabbit anti-flucloxacillin antibody diluted to 1:2000 in 2.5% blocking buffer and left to rock overnight

at 4°C. The thiazolidine ring has been hypothesized as the site of antibody detection in our formed β-lactam conjugates (figure 3.7a) apart from flucloxacillin conjugates where the masking of the thiazolidine ring by the side chain (figure 3.7b) suggests that the flucloxacillin-specific antibody is side chain specific.

v. Membrane development. Excess primary antibody was washed off the membrane with four 5 minute washes using TST. The membrane was transferred into goat anti-mouse antibody diluted to 1:10,000 in 2.5 % blocking buffer (2.5 g milk in 100ml TST) and left to incubate while rocking slowly for 1 hr at room temperature. After incubation, excess secondary antibody was washed off with four 5 minute washes with TST. Equal amounts of the brown and white developer (ECL) were combined and a total of 2 ml was used per blot. The blot was removed from the secondary antibody and dabbed to remove excess TST and laid facing up on a square of cling film. ECL was added in a manner that ensured it covered the whole blot and was left on for 1 minute. The excess ECL was dabbed off and wrapped by the cling film. With more than one blot wrapping was performed individually, while ensuring there were no air bubbles. The wraps were then secured in a cassette and taken into the dark room for exposure, taking care not to expose the film to any light beforehand. In the dark room the blots were exposed to the film(s) for varying durations after which the film was immersed in the developer (brown) for one minute, and fixer (yellow) for another minute then washed thoroughly with water to prevent the film from fogging. The film was then left to dry following which scanning was performed.

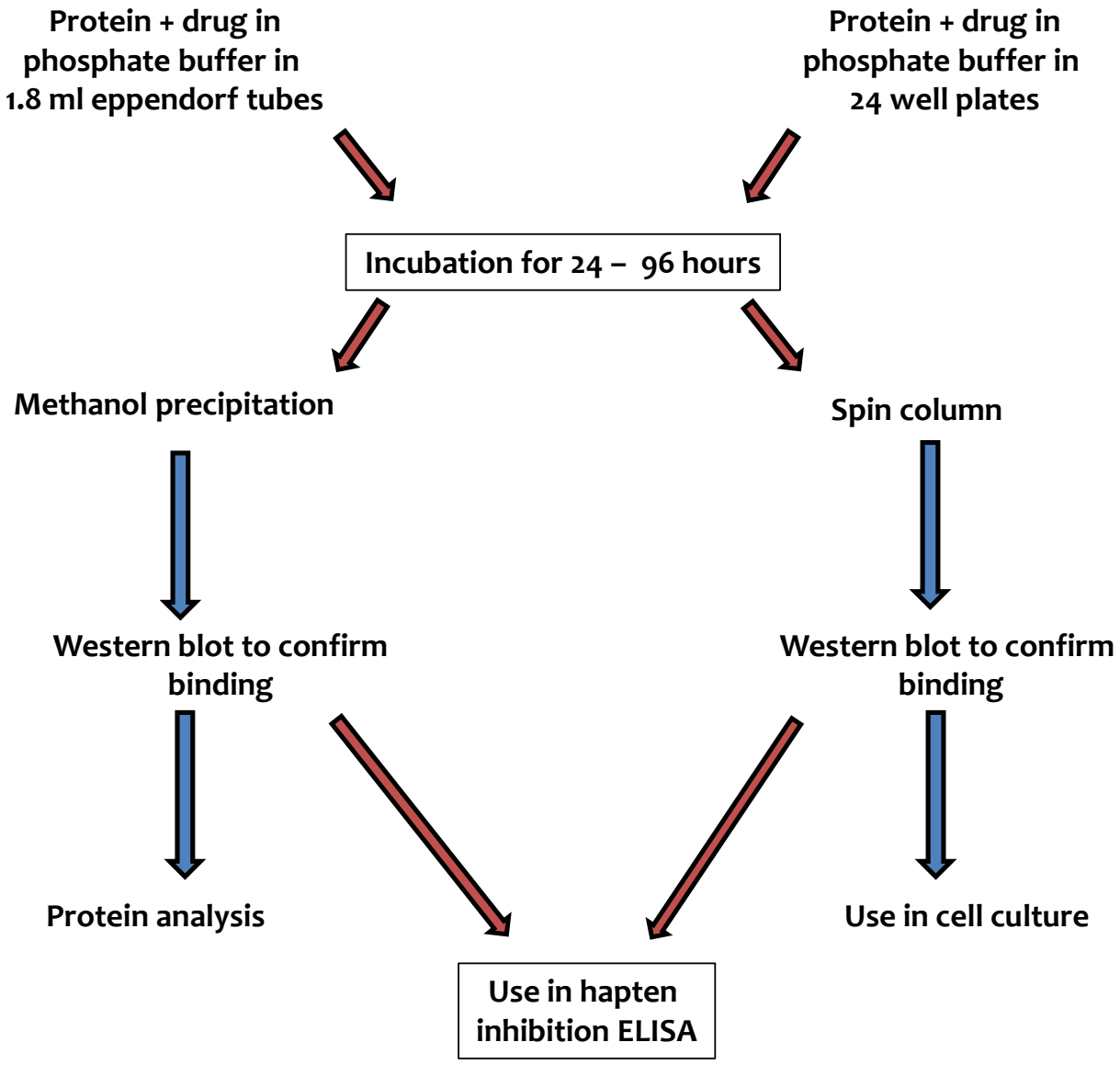


Figure 2.7 Schematic diagram of conjugate analysis and application. Figure shows the generation of protein conjugates, confirmation of adduction and their downstream applications.

2.3.2.2 Coomassie stain

Following the electrophoretic separation of proteins onto the gel, the gel was transferred into a dish containing gel fix solution (40 % methanol, 7 % acetic acid) and kept rocking for 1 hr. After fixing the gel fix solution was discarded and coomassie brilliant stain mixed with ethanol at a ratio 4:1 was added to the dish. After rocking for 1 – 2 hr when one could visualize the bands, the coomassie blue solution was discarded and the gel washed for one minute with a solution of 25 % ethanol and 7 % acetic acid to de-stain the gel. After washing, the gel was kept in 25 % ethanol overnight. The gel was scanned for use as a loading control or the bands detected were excised for LC-MS analysis.

2.3.3 Mass spectrometric analysis

2.3.3.1 Tryptic digests for mass spectrometric analysis

i. Tryptic digest: 100 mM of dithiothreitol (DTT) and 550 mM iodoacetamide (IODO) were prepared (0.0154 g DTT in 1 ml PO_4, 0.102 g IODO in 1 ml phosphate buffer). 10 µl of DTT was added to 100 µl of the samples. This mixture was vortexed and left at room temperature for 15 minutes. 1 ml of phosphate buffer was added to the already weighed out IODO from which 10 µl was removed and added to the sample. This mixture was vortexed again and left at room temperature for 15 minutes. 900 µl of methanol was added to the 100 µl sample and this mixture was centrifuged at 14000 x g for 15 minutes at 4°C. The methanol was discarded and 50 µl phosphate buffer added. Protein content was assessed using a simple Bradford assay and samples reconstituted to 5 mg/ml in 50 µl using 50 mM ammonium bicarbonate buffer. Trypsin at 1/20th to 1/100th of the sample volume was added. In this case 18 µl phosphate buffer was added to the vial, from which

5 µl was used for each of the three samples. This mixture was left at 37^{o}C in a dry incubator overnight.

ii. Zip Tip: Two 10 µl aliquots of the samples were transferred into new eppendorf tubes. 1 µl of 1 % trifluoroacetic (TFA) acid was added to acidify the sample. This sample was then vortexed and C18 Zip Tips (Millipore, Watford, U.K) wetted with 10 µl of 100 % acetonitrile (ACN) by pipetting up and down the tube 2 - 3 times. The tip was then made aqueous by washing 5 times with 0.1 % TFA, taking care to dispense into a waste bottle each time. The tips were then taken into the acid sample and pushed ten times up and down in the tube after which they were washed five times as previously described with 0.1% TFA. 10 µl of 75 % ACN + TFA was introduced into a new tube and labeled zip tipped. This 10 µl of 75 % ACN + TFA was drawn up with the Zip Tip, then taken into the sample and drawn up and down seven times up to the white line. On the last pipette all the sample was ejected into the tube labeled zip-tipped. The elution was now ready for MALDI analysis. The samples are also dried for 15 minutes in a speed vac with the lids open after which they were ready for LC-MS analysis.

2.3.3.2 Matrix assisted laser desorption ionisation (MALDI)

MATRIX and sample were prepared and each vortexed before use. 0.8 µl of the trypsinized and desalted sample was placed on a MALDI plate spot. 0.8 µl of the MATRIX was then transferred onto the sample. Calibration was achieved by the addition of 0.8µl of the calibration control which already contains matrix. This was placed in between the sample numbers The MALDI plate was then allowed to dry before being loaded into the mass spectrophotometer. When the desired number of hits and traces had been accumulated the data were collected using voyager control panel software.

2.3.3.3 Multiple Reaction Monitoring (MRM) characterization of β-lactam albumin binding

Samples were reconstituted in 2% acetonitrile (ACN)/0.1% formic acid (v/v), and aliquots of 2.4 – 5 pmol were delivered into a QTRAP 5500 hybrid quadrupole-linear ion trap mass spectrometer (ABSciex) by automated in-line LC (U3000 HPLC System, 5 mm C18 nano-precolumn and 75 µm × 15 cm C_{18} PepMap column; Dionex) via a 10-µm inner diameter PicoTip (New Objective). A gradient from 2% ACN/0.1% formic acid (v/v) to 50% ACN/0.1% formic acid (v/v) in 70 min was applied at a flow rate of 300 nl/min. The ionspray potential was set to 2200–3500 V, the nebulizer gas to 18, and the interface heater to 150°C. Multiple reaction monitoring (MRM) transitions specific for drug-modified peptides were selected as follows: the mass/charge ratio (m/z) values were calculated for all possible peptides with a missed cleavage at a lysine residue; to these were added the mass of the appropriate hapten (cyclised Piperacillin, 517 atomic mass units [amu]; hydrolysed Piperacillin, 535 amu; Amoxicillin, 365 amu; Amoxicillin with loss of NH_2, 349 amu; Benzyl Penicillin, 334 amu; Flucloxacillin, 453 amu; Penicillin V, 350 amu); the parent ion masses were then paired with a fragment mass of 160 ([M+H]+ of cleaved thiazolidine ring present in all of the haptens) and/or a fragment mass of 106 ([M+H]+ of cleaved benzylamine group of a Piperacillin haptens). See figure 4.2c for schematic showing the mechanism of adduct (primary and desethyl) generation with cyclised and hydrolysed products.

MRM transitions were acquired at unit resolution in both the Q1 and Q3 quadrupoles to maximize specificity, they were optimized for collision energy and collision cell exit potential, and the dwell time was 20 ms. MRM survey scans were used to trigger enhanced product ion MS/MS scans of drug-modified peptides, with Q1 set to unit

resolution, dynamic fill selected, and dynamic exclusion for 20 s. Total ion counts were determined from a second aliquot of each sample analysed by conventional LC tandem MS and were used to normalize sample loading on column. MRM peak areas were determined by MultiQuant 1.2 software (ABSciex).

A semi-quantitative analysis of covalent modification at multiple sites within BSA was performed. This led to the construction of epitope profiles for the comparison of individual modified peptides across treatments (eg different drug concentrations) but did not allow the abundance of one modified peptide to be compared to another modified peptide because of the difference in basal ionisation efficiency.

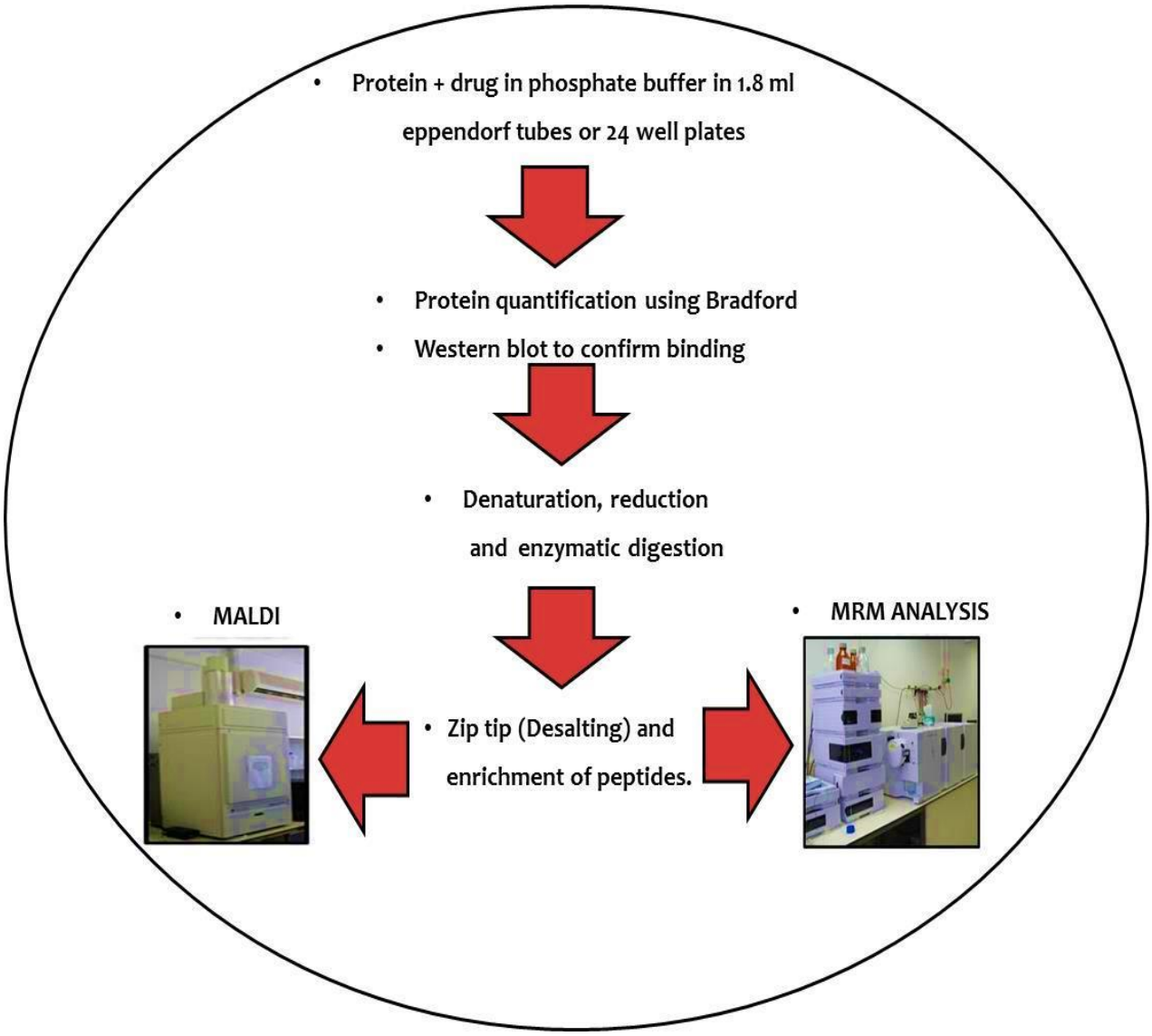


Figure 2.8 Schematic for sample preparation for mass spectrometric analysis.

2.4 Prospective study of piperacillin hypersensitive patients with CF

Piperacillin allergic and tolerant patients were recruited from the adult cystic fibrosis unit in Leeds. Blood samples were collected from these patients at 4 different time points; prior to piperacillin administration, midway into the treatment course, at the end of the

course and a prospective sample after a few months. 60 ml of blood at the beginning of the course and 12 ml for follow-up samples was collected into 10 ml tubes and isolated according to the method in section 2.1.2. PBMC and plasma were collected from each patient sample. A LTT was performed on each sample to assess the hypersensitivity status according to the method used in section 2.1.3. Plasma samples were used to perform total IgG ELISAs according to the method used in section 2.2.3.1, and ELISA for the detection of piperacillin-specific antibody according to the method used in section 2.2.3.2.

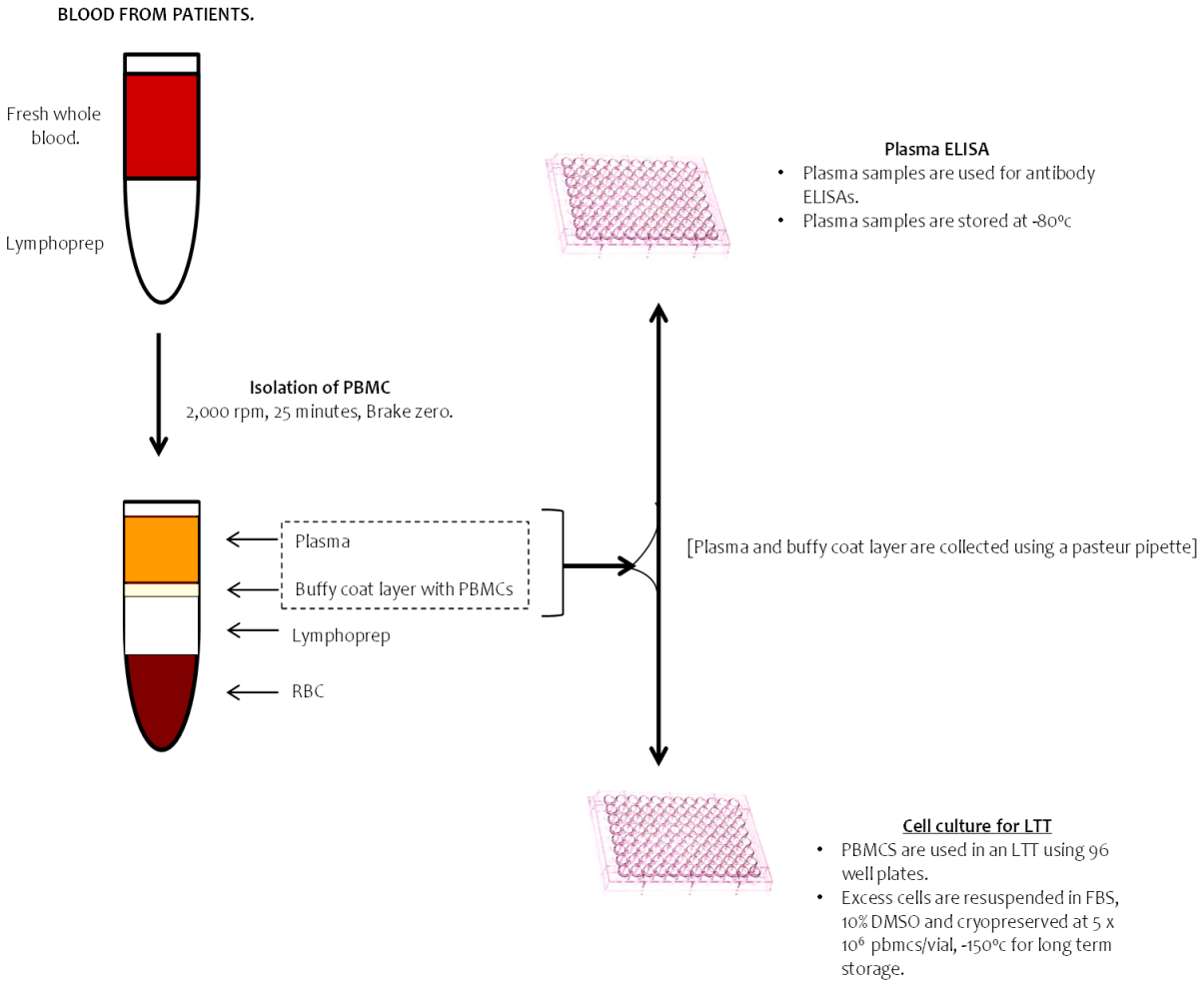


Figure 2.9 Schematic diagram for piperacillin tolerant and hypersensitive patient sample collection, isolation and analysis.

2.5 Statistical analysis

Experiments were performed in triplicate and replicated on several occasions. Data are presented as mean ± standard deviation of the mean (SD) unless otherwise stated. Normality of data was assessed with the Shapiro Wilkes test. The unpaired Student's *t* test at 95% confidence interval (CI), the Mann-Whitney or the Wilcoxon signed rank test were used to assess the significance of any differences in the data compared to appropriate controls. A two-sided *P* value of ≤ 0.05 was considered to be statistically significant.

CHAPTER 3

DETECTION OF THE B-CELL RESPONSE, AND DRUG-SPECIFIC IgG IN PATIENTS WITH PIPERACILLIN HYPERSENSITIVITY

Table of contents

Figures

Tables

3.0 Introduction

B-cell activation and differentiation brought about by protein or non-protein antigens, lipids, sugars or DNA occurs in secondary lymphoid tissues. Protein responses normally require the participation of T helper (Th) cells specific for the antigen. T-cell dependent B-cell responses are initiated at the edges of the follicles where B cells present the antigen to T-cells (Engel, Gomez-Puerta et al. 2011). The mainstay of the humoral immune response is anchored not only on the support rendered by the Th cells, but also on molecular events. The origin of mature B cells in the germinal centre can be traced to events such as cell differentiation, affinity maturation and class switch recombination (Han, Wang et al. 2014), which are controlled by molecules including lipopolysaccharide (LPS), cytosine phosphate guanosine-deoxyribonucleic acid (Cpg-DNA), or Polyinosinic acid:polycytidylic acid (poly(I:C)), a chemical agonist of TLR-3. All of these molecules elicit polyclonal B cell activation via toll-like receptors (Hoffmann, Galanos et al. 1977, Bauer, Heeg et al. 1999, Marshall-Clarke, Downes et al. 2007).

B-cells are immunoglobulin producing cells. This property of B cells, though indicative of the protective role they play in the event of infection, also features prominently in the development and sustenance of inflammatory responses in certain disease conditions and hypersensitivity responses to drugs and environmental allergens. This is thought to contribute significantly to the subsequent presentation of clinical symptoms (Smits 2012), confirmed earlier via the assessment of antibody levels and specific antibodies to particular antigens which arise as a consequence of antigen induced cell activation an important part in the diagnosis of various diseases (Greenwald, Burstein et al. 2006, Niewold, Harrison et al. 2007). However, in some instances further diagnostic tests are

required to reach a more definite conclusion as illustrated by HCV-specific IgG and IgM detection in acute and chronic hepatitis which was possible *in vivo* but only inducible in a minority of patients' *in vitro* (Quinti, Hassan et al. 1995). Furthermore, elevated levels of IgG and IgA, but not IgE, have been detected with three polymeric variants of diisocyanate hexamethylene diisocyanate (HDI), methylene diphenyl diisocyanate (MDI), and toluene diisocyanate (HDI, MDI, and TDI). These are chemical agents regularly employed in industry, but are capable of causing isocyanate-induced hypersensitivity pneumonitis, an acute inflammatory response which has an onset that ranges from 2 - 9 hrs after exposure. Additionally IgG has been implicated in type II and III allergy according to Gell and Coombs but has not lent itself to a single test to enable easy diagnosis (Dixon 2000). Nonetheless, data generated using the ELISA and ELISpot assays have shown serum antibody responses which were mainly of the IgG type, generated in DNA vaccinated mice that developed antigen-specific delayed type hypersensitivity (Zhang, Yang et al. 1999) and also in nude mice grafted with thymic epithelium (Jordan, Chapman et al. 1990).

In addition to their exclusivity with regard to the production of immunoglobulins, B cells are also known to express specific surface markers which have been introduced in chapter 1. Of primary importance is the pan B cell marker CD19 that promotes the survival and proliferation of mature B cells but plays a less understood role in immature B cells (Otero and Rickert 2003). This property serves to project CD19 as a suitable marker for B cell growth and proliferation. CD20 is also expressed on B-cells during all stages of development. Originally its expression was thought to be restricted to B-cells; however low levels of expression has recently been observed in a small population of $CD3^{+}CD20^{dim}$

T cells. Though earlier disputed in some quarters the presence of this subset is no longer in doubt (Hultin, Hausner et al. 1993, Palanichamy, Jahn et al. 2014). The development of memory B-cells with the ability to generate immunoglobulins rapidly in the secondary immune response has been defined by the enhanced expression of a CD19+CD27+ population (Agematsu 2000, Agematsu, Hokibara et al. 2000). This makes CD27+ an attractive marker to measure by flow cytometry to characterise the activation of B-cells.

The ability to activate B-cells isolated from human peripheral blood leading to their proliferation via induction by T cell derived mitogens (CD40 ligands) + IL-4, and Cpg-dna has been described previously (Bauer, Heeg et al. 1999, Huggins, Pellegrin et al. 2007, Carpenter, Mick et al. 2009). Epstein-Barr virus has been employed in the immortalisation of B lymphocytes (Wall, Henkel et al. 1995). This confers upon the normal lymphocytes the ability to proliferate indefinitely as has been originally reported (Miller 1982). EBV-immortalized lymphocytes still possess properties similar to normal B cells and they are thought to secrete IgG in certain circumstances (Kataoka, Tahara et al. 1997, Sugimoto, Furuichi et al. 1999). Novel methods have been established for the immortalization of antigen-specific human B cells for the generation of human monoclonal antibodies. Methods used by some researchers have involved EBV immortalisation and TLR9 stimulation, leading to the isolation of antibodies from B cell clones from a patient recovering from severe acute respiratory syndrome coronavirus (SARS-cov), which were specific for viral proteins. Others have utilised a different approach employing STAT5 overexpression for this purpose (Steinitz, Klein et al. 1977, Traggiai, Becker et al. 2004, Scheeren, van Geelen et al. 2011).

The mechanism of activation of T-cells by β-lactams in hypersensitive patients with cystic fibrosis has previously been reported and the cellular processes that underlie the structural specificity of β-lactam protein binding has also been described (El-Ghaiesh, Monshi et al. 2012). However the role of humoral processes specifically the mechanism of B cell activation and the roles of the B cell immunoglobulins in the disease state and drug hypersensitivity have not been defined. Thus, the main aims of this chapter were to;

- Develop cell culture methods for the detection of the B cell response in hypersensitive patients.
- Analyse IgG subclasses in hypersensitive patients.
- Develop antibody producing B cell lines.

3.1 Methods

The cell culture methods employed in this chapter include PBMC isolations (section 2.1.2), lymphocyte transformation tests (section 2.1.3), PBMC culture assays (section 21.4), B-cell separation and immortalization (sections 2.1.5 – 2.1.6), phenotypical characterization of PBMC populations using flow cytometry (section 2.2.2.1). Functional characterization of B cells (section 2.12), plasma and supernatants (sections 2.2.3.1–2.2.3.4). Other methods employed involved the preparation of β-lactam adducts (section 2.3.1), western blot analysis of formed adducts (section 2.3.2.1) and mass spectrometric analysis (2.3.3).

3.2 Results

3.2.1 Patient and volunteer characteristics

PBMC were isolated from 3 hypersensitive patients for the mechanistic studies described in this chapter. Table 3.2 describes the clinical characteristics of the patients and the nature of the reactions. For comparison, 3 patients with CF exposed to multiple piperacillin courses and 4 naïve healthy donors were used.

Table 3.1 Patient and volunteer characteristics. The table above shows the age and gender of hypersensitive and tolerant patients, the drugs they were treated with, the type of hypersensitive reaction, the time to onset of physical manifestations of hypersensitivity from the start of treatment and magnitude of the response to the lymphocyte transformation test.

	Patient (Cohort designation)	Age (years)/ Gender	Drug	Reaction	Time from treatment to reaction (Days)	LTT
1	Hypersensitive patient 1	18/M	Tazocin	MPE/Fevers	7	++
			Aztreonam	MPE	5	-
			Ceftazidime	Delayed angioedema	5	-
			Meropenem	MPE	1	+
2	Hypersensitive patient 2	19/M	Ceftazidime	MPE	5	-
			Tazocin	MPE/Fever	2	++
3	Hypersensitive patient 3	N.A	Piperacillin	MPE	6	++
			Tazocin	N.A	N.A	N.A

++ Highly positive LTT

+ Weakly positive LTT

— Negative LTT

N.A Not available.

3.2.2 Lymphocyte transformation tests (LTT)

Preliminary assays were carried out to confirm the suspected hypersensitivity status of patients using blood samples obtained from the regional adult CF unit in Leeds. The lymphocyte transformation test was carried out using PBMC from naïve volunteers, hypersensitive and tolerant patient samples. A stimulation index (S.I) greater than 2 was taken as a positive result for hypersensitivity to piperacillin. Following isolation from blood, PBMC were exposed to piperacillin 0.125-8 mM, and maximal proliferation was seen at 1 mM for patients 1 and 2, and at 2 mM for patient 3. Hypersensitivity was regarded as the production of a SI > 2. The response observed with piperacillin 1 mM was significantly stronger and more consistent across all the samples than those of other concentrations; thus, this dose was selected for all subsequent cell culture assays. PBMCs from piperacillin naïve volunteers and tolerant patients were not activated in response to piperacillin treatment (d-i).

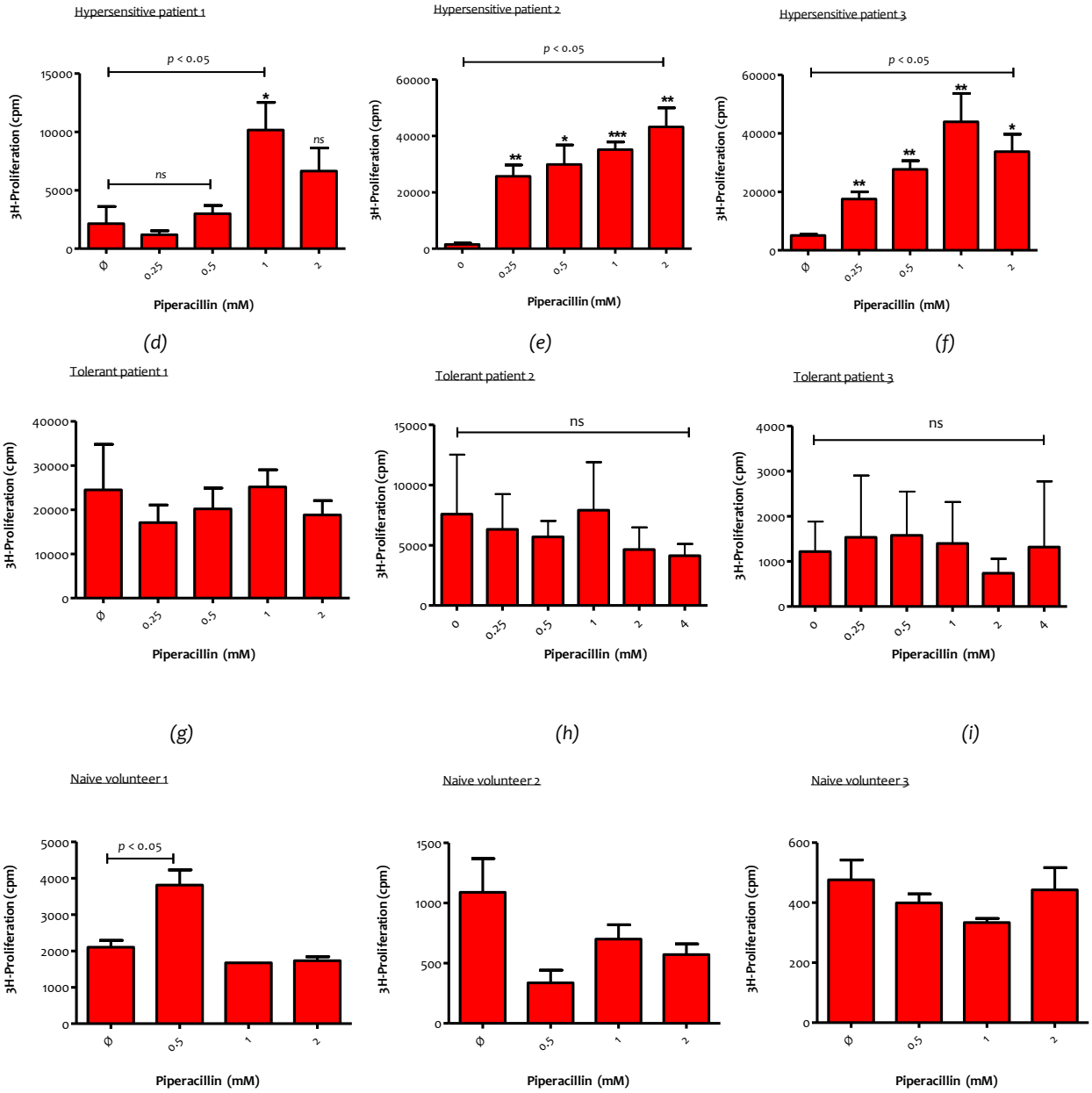


Figure 3.1 LTT from naïve volunteers, piperacillin hypersensitive and tolerant patients. PBMCs (1.5×10^5 cells in 100 µL) from hypersensitive patients (a - c) tolerant patients (d - f) and drug naïve volunteers (g-i) were incubated with graded concentrations of piperacillin (0.125-8 mM in 100 µL) in 96-well U-bottom plates. Plates were incubated at 37°C under an atmosphere of 5% CO_2 for five days. [3H]-thymidine (0.5 µCi/well) was added for the final 16 hours of incubation and T-cell proliferation measured using scintillation counting in a Beta counter. The data were analysed by Students T-test with $p < 0.05$ considered significant (* denotes $p < 0.05$ ** denotes $p < 0.01$; *** denotes $p < 0.001$).

3.2.3 Phenotype of B-cells ($CD19^+CD27^+$) following mitogen and piperacillin stimulation

PBMCs isolated from piperacillin naïve volunteers, and piperacillin tolerant and hypersensitive patients with CF were cultured with mitogen or piperacillin (2 mM) for five days after which the resultant phenotype was assessed. Compared to the untreated (29.2 ± 2.9%) and positive (46.9 ± 14.1%) controls, the piperacillin treated groups (24.9 ± 4.2%) in the naïve volunteers [figure 3.2] did not show an expansion in the memory B cell (CD19+CD27+) population [figure 3.2]. A similar result was obtained with PBMC from tolerant patients with no difference observed between the drug-treated and untreated control (untreated control = 8.5 ± 0.2%; Piperacillin = 11.6 ± 6.3%; and Cpg-dna = 15.6 ± 3.8% ; $p > 0.05$). In contrast, a significant increase in the expression of memory B cells was observed with the piperacillin-treated PBMC from hypersensitive patients when the drug treated and untreated control cells were compared ($p \leq 0.05$). The number of cells staining positive for CD19CD27 increased from 18.3 ± 5.7% to 28.5 ± 1.27% following drug treatment. 30.0 ± 6.35% cells stained positive for CD19CD27 after exposure to the mitogen control.

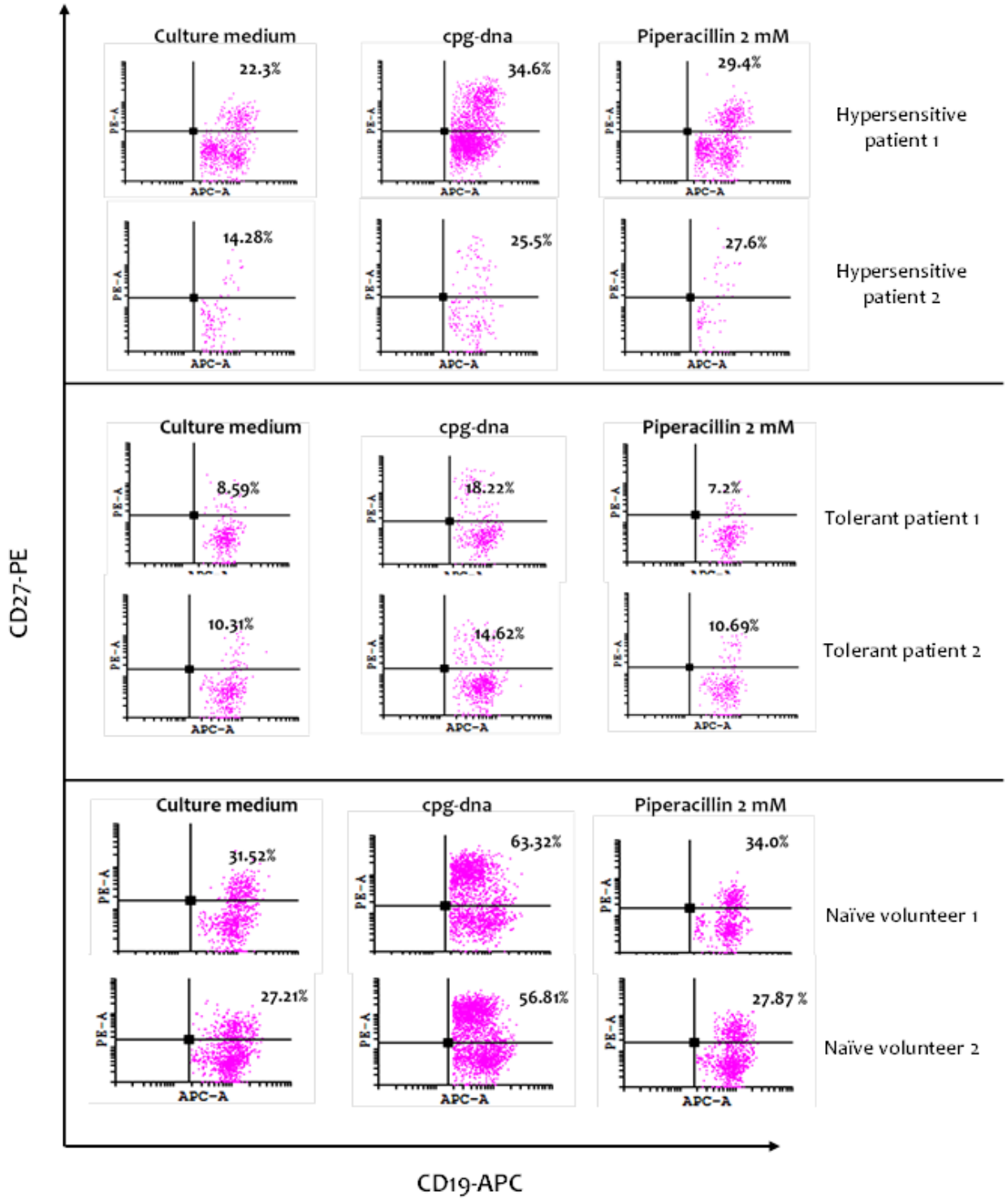


Figure 3.2.a B-cell phenotyping for CD19+CD27+ cell surface expression from piperacillin naïve volunteers and piperacillin tolerant and hypersensitive patients. PBMCs were incubated in medium or stimulated with CpG- DNA or piperacillin for 5 days. PBMC suspensions (200 µL) were incubated with CD3-FITC, CD19-APC and CD27-PE immunoflourescent antibodies for 20 minutes at 4°C. Cells were washed and surface staining by the fluorescent markers was measured by flow cytometry. Data were expressed as dot plots to obtain percentage of cells in each quadrant (with n=3) for each treatment condition.

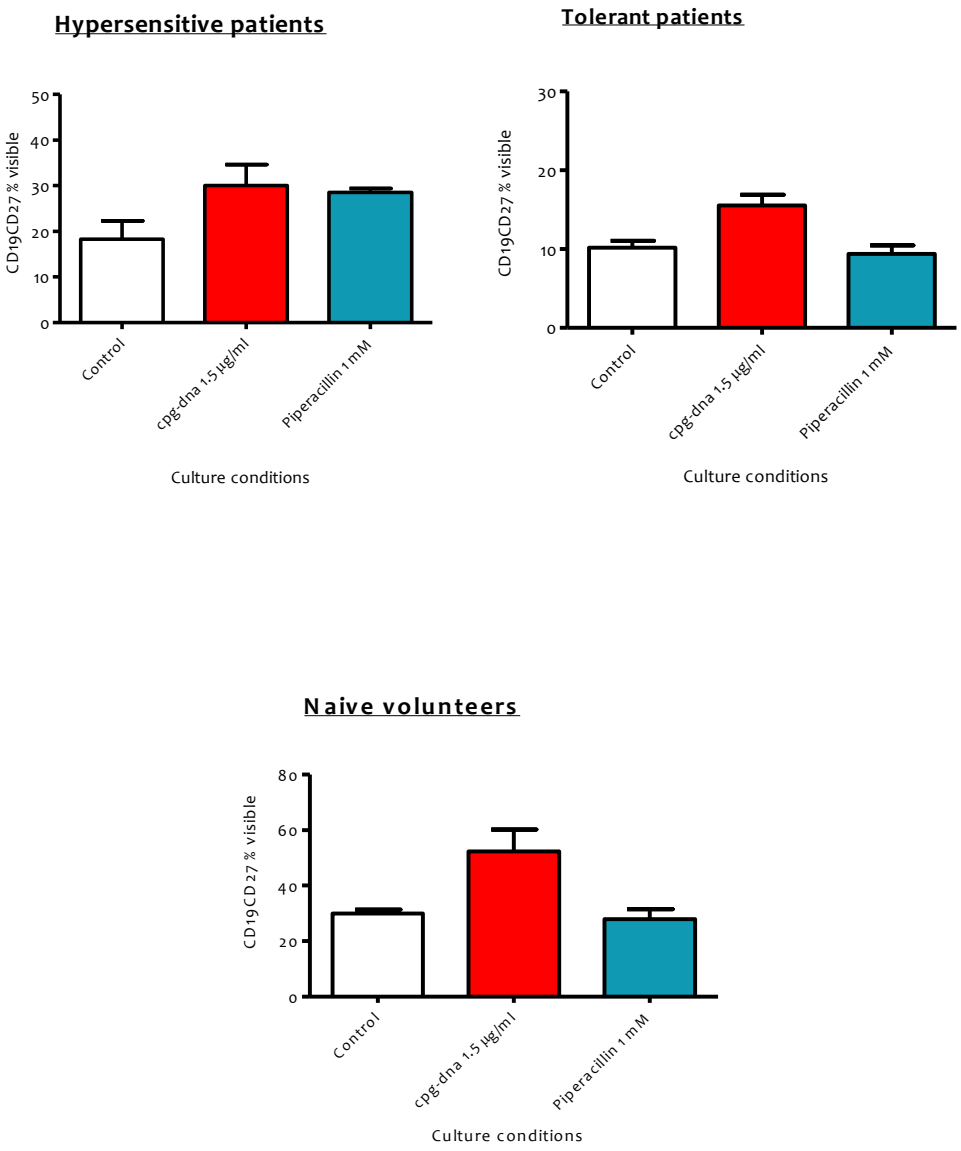


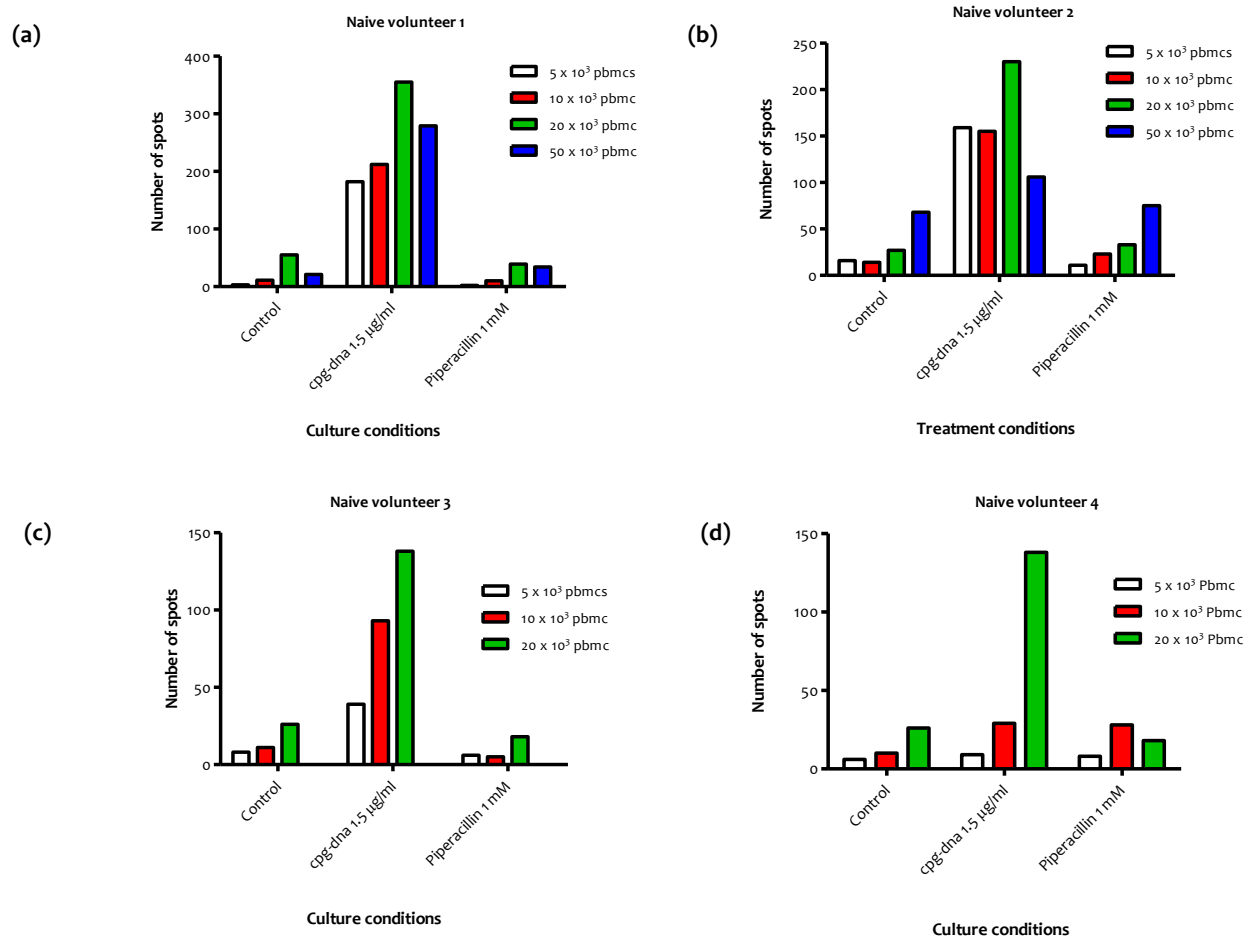
Figure 3.2.b B-cell phenotyping for CD19+CD27+ cell surface expression from piperacillin naïve volunteers and piperacillin tolerant and hypersensitive patients. Graphical representation of the data in Figure 3.2a showing CD19CD27 expression on from naïve volunteers, tolerant and hypersensitive patients. The data were analysed by Students T test with $p < 0.05$ considered significant (* denotes $p < 0.05$;** denotes $p < 0.01$).

3.2.4 Optimization of an ELISpot assay for determination of IgG antibody secretion as a marker for antigen-specific B cell activation

Using PBMC from piperacillin naïve volunteers, preliminary experiments were carried out to determine the optimum conditions under which *in vitro* secretion of IgG from isolated B-cells could be pursued.

The ELISpot plates were pre-coated with anti-human IgG according to the manufacturer's instructions and incubated overnight at 4°C. PBMCs were harvested from 24 well plates after 120 hour incubation, transferred to each well of the ELISpot plate and incubated at 37°C, 5% CO_2. After 48 hours the ELISpot plates were developed according to the manufacturer's instructions, and the wells left out to air dry. Spots were visualised using an AID ELISpot reader.

Figure 3.3A-B and E show results of IgG ELISpots conducted with PBMC from different drug naïve healthy donors cultured at different cell numbers of 4 x 10^3, 10 x 10^3, 20 x 10^3 and 50 x 10^3 PBMC. Figure 3.3 C-D show separate experiments where 5 x 10^3, 10 x 10^3, and 20 x 10^3 PBMCS were used. An increase in the number of IgG secreting cells was observed at each cell number when the mitogen-treated cells were compared to the negative control. As expected piperacillin did not increase IgG secretion. Experiments conducted with 20 x 10^3 PBMCS produced the most consistent results (both in the magnitude of spots and consistency across all the samples) and as such, this cell number was used in all subsequent experiments using PBMC from piperacillin naïve, tolerant and allergic patients.



(e)

Culture conditions	Naïve volunteer 1			Naïve volunteer 2			Naïve volunteer 3			Naïve volunteer 4		
	5,000 PBMC	10,000 PBMC	20,000 PBMC	5,000 PBMC	10,000 PBMC	20,000 PBMC	5,000 PBMC	10,000 PBMC	20,000 PBMC	5,000 PBMC	10,000 PBMC	20,000 PBMC
Control												
Cpg-dna 1.5µg/ml												
Pip 1 mM												

Figure 3.3 Optimization of the cell concentration needed for the detection of total IgG secretion by PBMCs from naïve volunteers. 1 x 10^6 PBMCs were cultured in 24 well plates with piperacillin and cpg-dna for 5 days. ELISpot plates were pre-coated with anti-human IgG according to the manufacturer's instructions and incubated overnight at 4°C. After washing PBMC's were harvested and 5 x 10^3, 10 x 10^3, 20 x 10^3 transferred to each well and incubated for 48 hours. ELISpot plates were developed according to the manufacturer's instructions, and the wells left to air dry. Spots were counted using an AID ELISpot reader. Results from 4 naïve volunteers (a) - (d) graphical representation of spot counts, (e) pictures of ELISpot wells.

Following the determination of optimum cell numbers required for the ELISpot assay, ELISpot plates were pre-coated with anti-human IgG according to the manufacturer's instructions and incubated overnight at 4°C. PBMCs (20 x 10^3) from the three patient groups were harvested from 24 well plates after 120 hour incubation, were transferred onto each well of the ELISpot plate and incubated for 48 hours. After washing ELISpot plates were developed according to the manufacturer's instructions, and the wells left out to air dry. Spots were visualised using an AID ELISpot reader.

Figure 3.4 A - I show results of IgG ELISpots conducted with PBMC from piperacillin naïve volunteers with tolerant and hypersensitive patients cultured at cell numbers of 20 x 10^3. A single measurement was taken for each sample for the various treatment groups. An increase in the number of IgG secreting cells was observed with all groups when mitogen-treated cells were compared to the negative control. Piperacillin treated hypersensitive patients PBMC also showed an increase in IgG secretion.

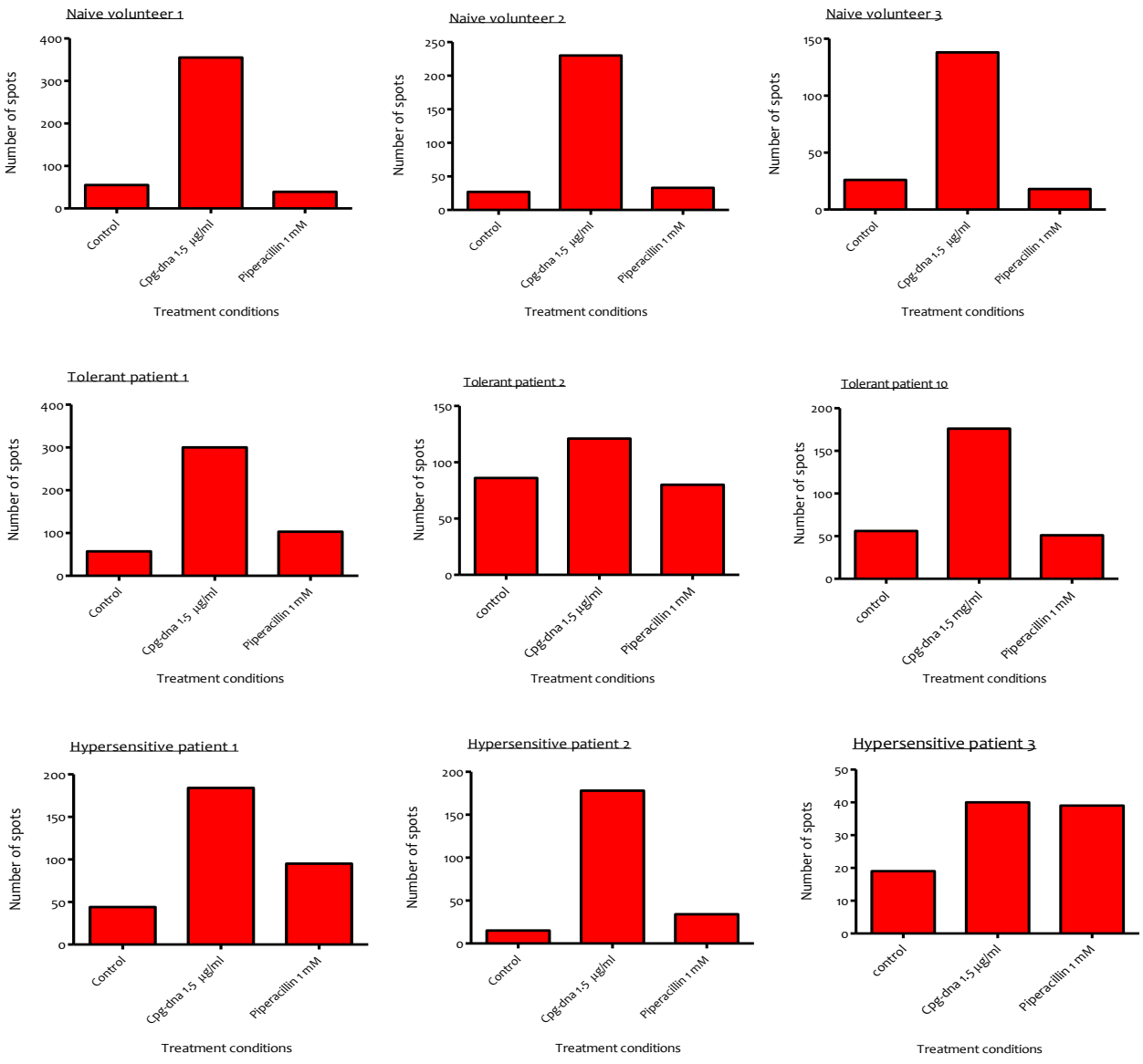


Figure 3.4.1 Total IgG secretion from naïve volunteers, piperacillin hypersensitive and tolerant patients. 1 x 10^6 PBMCs were cultured in 24 well plates with piperacillin (1 mM) and cpg-dna (1.5µg/ml) for 5 days. The ELISpot plates were pre-coated with anti-human IgG according to the manufacturer's instructions and incubated overnight at 4°C. PBMCs were harvested and 5 x 10^4 transferred to each well and incubated for 48 hours. ELISpot plates were developed according to the manufacturer's instructions, and the wells left to air dry. Data were analysed using an AID ELISpot machine.

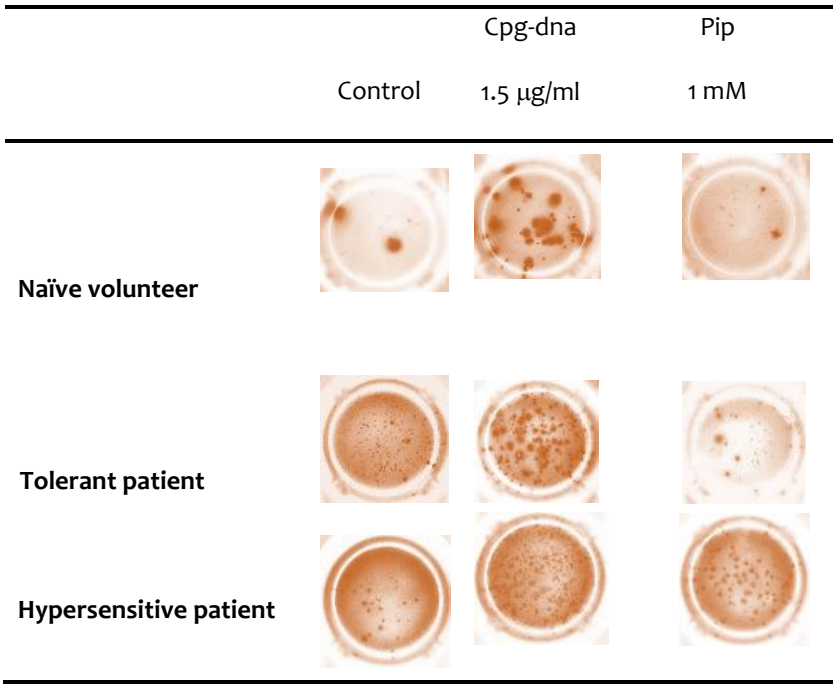


Figure 3.4.2 Total IgG secretion from naïve volunteers, piperacillin hypersensitive and tolerant patients. Spots were visualised using an AID ELISpot reader. Representative images from an ELISpot plate of naïve volunteer, tolerant and hypersensitive patient.

3.2.5 ELISA for total IgG secretion from piperacillin and mitogen-treated PBMC

High binding 96 well plates were pre-coated with anti-human IgG according to the manufacturer's instructions and incubated overnight at 4°C. Samples were added in a 1:10 dilution and the ELISA carried out as described in chapter 2.

Mitogen-treatment of PBMC was associated with a significant increase in total IgG secretion in all but one patient from all treatment groups. An increase in IgG levels was not detected with supernatants from one naïve volunteer (figure 3.5). In contrast IgG secretion from piperacillin-treated PBMC was much more restricted. Small but significant increases in IgG were only observed with PBMC from 1 out of 3 hypersensitive patients with $p = 0.027$ (figure 3.5)

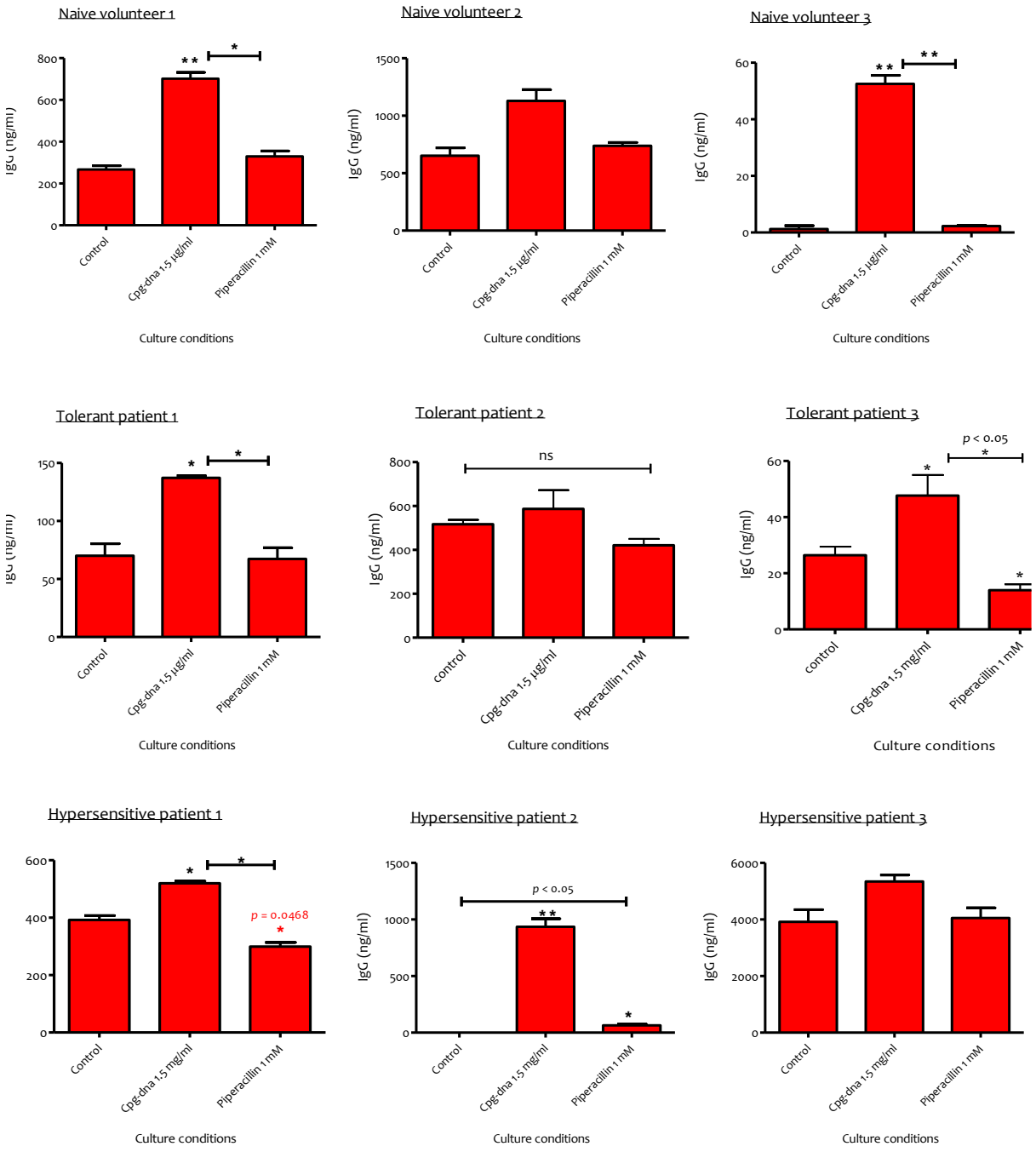


Figure 3.5 Total IgG secretion in naïve volunteers, tolerant and hypersensitive patient supernatants. 1 x 10^6 PBMCs from naïve volunteers, tolerant and allergic patients were cultured with piperacillin (1 mM) and cpg-dna (1.5 µg/ml) in 24 well plates for 5 days. Flat bottomed high binding 96 well plates were pre-coated with anti-human IgG according to the manufacturers' instructions and incubated overnight at 4°C. After washing 100 µl cell culture supernatants diluted at a ratio of 1:10 (Supernatant:diluent) was added to each well and incubated for 1 hr. The presence of drug specific antibodies was detected by anti-IgG HRP conjugated antibody and TMB substrate. The reaction was stopped with 0.18M H_2SO_4 and absorbance measured at 470nm using a spectrophotometer. The data was analysed by Students T test with p<0.05 considered significant (* denotes $p < 0.05$;** denotes $p < 0.01$).

3.2.6 *In vitro* generation of HSA:Piperacillin adducts and characterisation by mass spectrometry

The presence of antidrug antibodies in certain penicillin hypersensitive patients has been described previously using piperacillin-modified HSA as an antigen (Christie, Coleman et al. 1988). To assess the quantity of piperacillin-specific IgG in plasma of volunteer and patient groups, the initial step required the generation of protein conjugated to the drug molecule at sufficient levels for antibody binding, but generated via a method that did not result in significant non-specific binding.

Initially, SDS-PAGE western blot analysis was carried out to confirm the binding of piperacillin to HSA, at HSA:Piperacillin binding ratio of 1:50 after 96 hours incubation (figure 3.6). Adduct formation was confirmed at this point. Notwithstanding the cleavage of the β-lactam rings, the monoclonal antibodies (MABs) raised against the benzylpenicilloyl group have been found to recognize at least three separate epitopes. Specific sites identified include the side chain, the new antigenic determinant and the thiazolidine ring of penicillin dependent on the nature of the MAB antibody raised (de Haan, de Jonge et al. 1985). Similar to benzylpenicillin the piperacillin hapten adopts an adduct structure with the thiazolidine ring exposed when bound covalently to lysine groups on protein(figure 3.7b). Thus, the MAB that recognises the thiazolidine ring was used to visualize the piperacillin protein binding by western blotting. In contrast, the thiazolidine ring of flucloxacillin is masked when bound covalently to protein. Thus, an antibody raised in rabbit by Van Pelt (Carey and van Pelt 2005) through immunisation with a flucloxacillin-rabbit serum albumin conjugate was used to study flucloxacillin protein binding

This adduct was assessed for the nature of amino acid modification post conjugation by mass spectrometry, analysis of which showed that covalent binding occurred to specific lysine residues in the protein. This occurs via the spontaneous opening of the β-lactam ring leading to the formation of penicilloyl conjugates. Fifteen penicilloylated lysine residues were detected at a drug-protein molar ratio of 50:1 (Table 3.2). The piperacillin adducts undergo fragmentation into cyclised and hydrolysed forms (figure 3.7 a). These forms are represented in the MS/MS spectra of one of the tryptic peptides ^{182}LDELRDEGK*ASSAK^{195} as an example. This peptide bears a covalent modification of Lys190 with a penicilloyl hapten (Figure 3.7 c). Confirmatory evidence to back up the modification stems from the presence of the characteristic fragment ions at *m/z* 160 and 143, which are indicative of a cleavage of the thiazolidine ring and anticipated fragmentation of the piperacillin hapten.

(a)

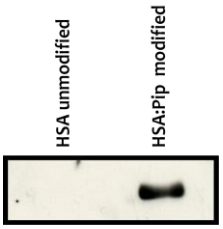


Figure 3.6. SDS-PAGE confirmation of HSA:Piperacillin binding. Protein and drug were mixed at a molar ratio of 1:50 then incubated for 96 hours at 37°C (ref). Adducts were then precipitated with methanol, dried and dissolved in RPMI 1640. Samples (5 µg/ml) were run on SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was blocked with 2.5 % milk in TST and incubated overnight at 4°C with a monoclonal mouse anti-penicillin antibody. After washing, the membrane was incubated with goat anti-mouse HRP-conjugated secondary antibody prior to ECL development with photographic film.

	LYSINE	PEPTIDE
1.	212	AFK*AWAVAR
2.	195	ASSAK*QR
3.	541	ATK*EQLK
4.	4	DAHK*SEVAHR
5.	545	EQLK*AVMDDFAAFVEK
6.	159	HPYFYAPELLFFAK*R
7.	525	K*QTALVELVK
8.	137	K*YLYEIAR
9.	12	FK*DLGEENFK
10	351	LAK*TYETTLEK
11.	190	LDELRDEGK*ASSAK
12.	199	LK*CASLQK
13.	432	NLGK*VGSK
14.	436	VGSK*CCK
15.	162	YK*AAFTECCQAADK

***** *Indicates site of modification*

Table 3.2 Penicilloylated lysine residues due to HSA:piperacillin adduction. The binding of piperacillin to HSA leads to the modification of lysine residues in HSA. Mass spectrophotometric characterization of the formed adduct was carried out to determine the extent of residue modification. Adducts were denatured and treated enzymatically with trypsin. Desalting and enrichment of peptides was carried out prior to mass spectrophotometric analysis.

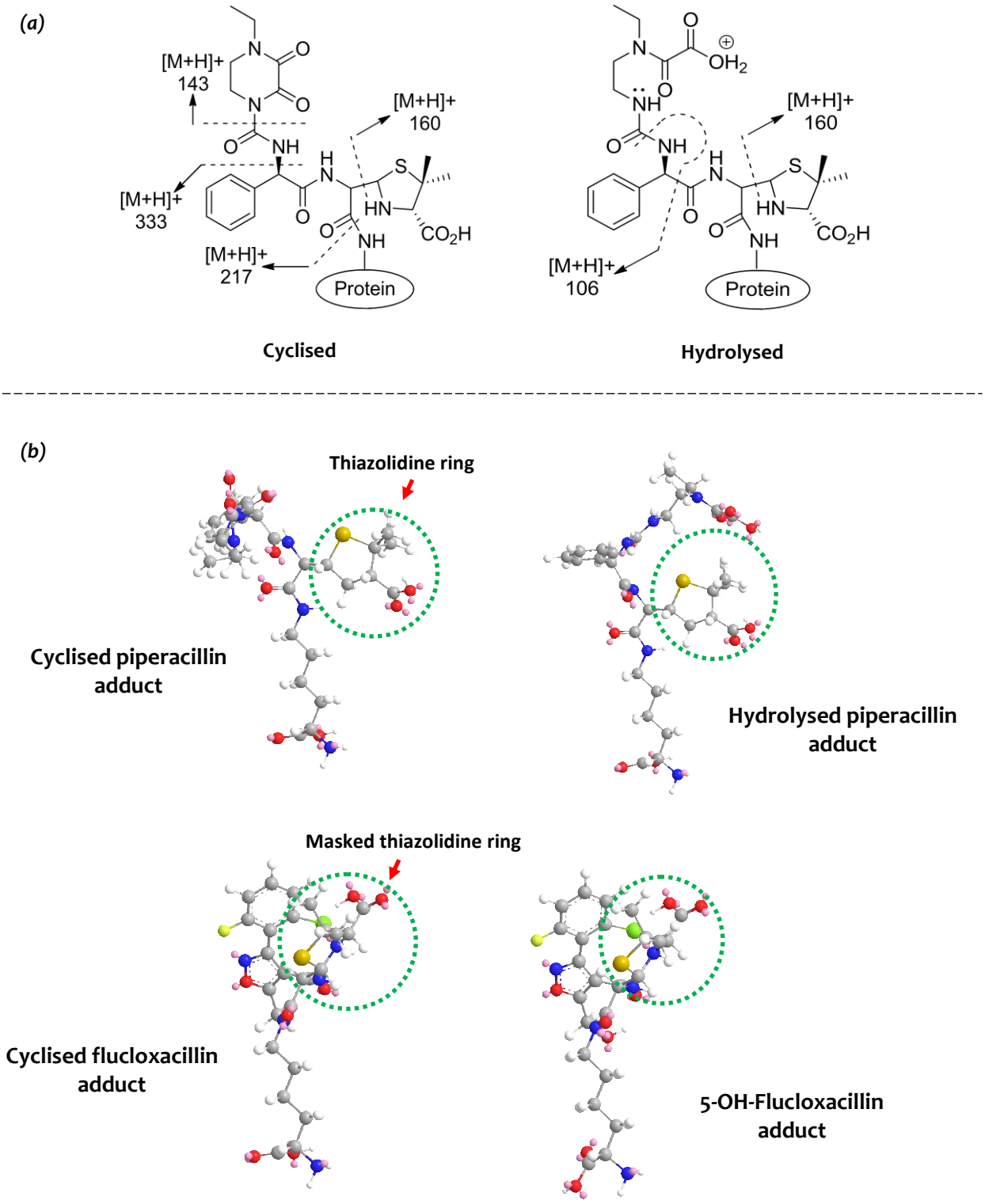
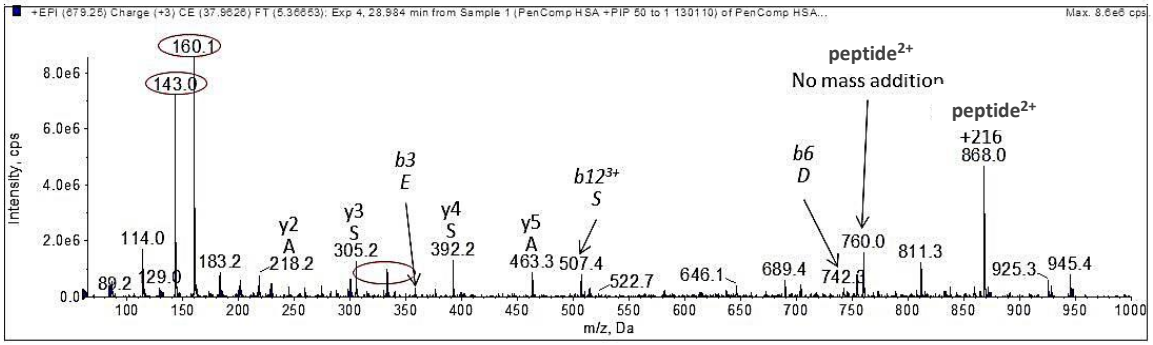


Figure 3.7 Chemical structures of cyclised and hydrolysed forms of the modified piperacillin hapten bound covalently to a tryptic peptide derived from HSA. (a) Chemical structure of cyclised and hydrolysed forms of HSA:Piperacillin conjugates and (b) Crystal structure models of cyclised and hydrolysed piperacillin and flucloxacillin adducts showing the conformational change that leads to the masking of the thiazolidine ring by the side chain.

(c)

Cyclised

^{182}LDELRDEGK*ASSAK^{195} + cyclised PIP (3+ 679.25, M*=2034.7, M=1517.8, Δ=516.9)



Hydrolysed

^{182}LDELRDEGK*ASSAK^{195} + hydrolysed PIP (3+ 685.25, M*=2052.7, M=1517.8, Δ=535)

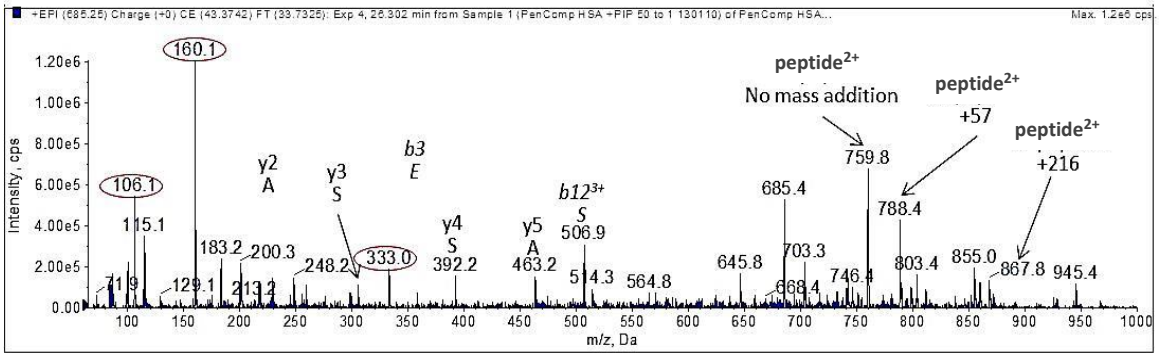


Figure 3.7c Chemical structures and MRM spectral images of cyclised and hydrolysed forms of the modified piperacillin hapten bound covalently to a tryptic peptide derived from HSA. Spectral images of MRM MS spectrometry analysis of cyclised and hydrolysed modification on LYS190 showing the presence of characteristic fragment ions at m/z 160 and 143 and 106.1 and 333.0.

3.2.7 Optimization of hapten inhibition ELISA to detect drug-specific IgG in hypersensitive patient plasma

The low level of IgG secretion exhibited by tolerant and hypersensitive patient PBMC culture supernatants suggested that it was unsuitable for the characterisation of drug-specific antibodies. Thus protein-drug adducts were prepared (figure 3.6) and plasma was used in an alternative approach to detect these antibodies. To optimize the assay different piperacillin-HSA adduct concentrations bound to plates was used for the detection of drug-specific IgG. Furthermore different dilutions of known hypersensitive patient plasma were applied to optimize the assay conditions (figure 3.8). Anti-piperacillin antibody was discernible; however, experiments were constantly hindered by the high levels of non-specific binding associated with adducts prepared using HSA (figure 3.8). This necessitated the preparation of adducts using alternative proteins in the generation of piperacillin antigens. These adducts were then compared with HSA adducts in the detection of IgG (figure 3.9) to assess their relative or not of non-specific binding.

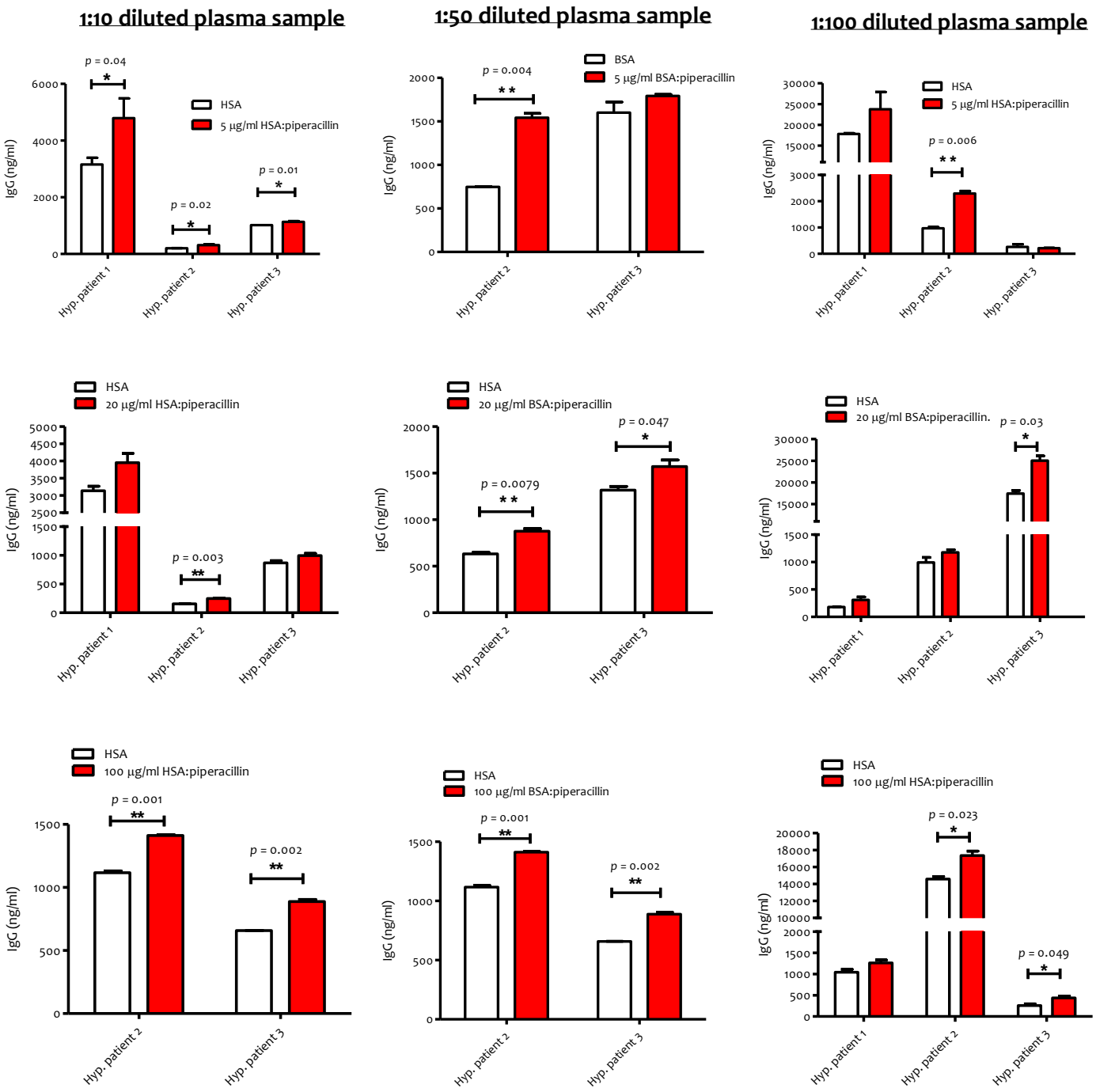


Figure 3.8. Optimization of the detection of anti-drug antibodies by ELISA. Three concentrations of unmodified BSA and piperacillin-modified HSA were used to coat flat bottomed high binding 96 well plates. After washing, 100 µl plasma from 3 different hypersensitive patients was added at 3 different dilutions and incubated for 1 hr. The presence of drug-specific antibodies was detected by anti-IgG HRP conjugated antibody and TMB substrate. The reaction was stopped with 0.18M H_2SO_4. The data were analysed by Students T-test with $p < 0.05$ considered significant (* denotes $p < 0.05$;** denotes $p < 0.01$).

3.2.8 Formation of piperacillin adducts using human serum albumin (HSA), bovine serum albumin (BSA) and lysozyme (LYS) for the optimization of the IgG ELISA

Protein:Piperacillin adducts were generated with three proteins bovine serum albumin (BSA), human serum albumin (HSA), and lysozyme (LYS) using methods previously described in section 2.16. SDS-PAGE Western blot analysis was carried out to confirm the protein:piperacillin binding after a 96 hour incubation for, at protein:drug binding ratio of 1:50 (Figure 3.9). Piperacillin modification of all proteins was detected.

Effects of the various unmodified and drug-modified proteins on different parameters of the ELISA assay including; diluents used, PBMC culture supernatants, and plasma were established. Unmodified BSA, HSA, and LYS all showed negligible absorbance levels [figure 3.10 (a)] with the sample diluent. Supernatants produced high backgrounds with HSA [figure 3.10 (b)]. Plasma elicited high backgrounds with both HSA and LYS with only BSA yielding low levels that might be suitable for the establishment of an assay to detect piperacillin-specific IgG [figure 3.10 (c) and (d)].

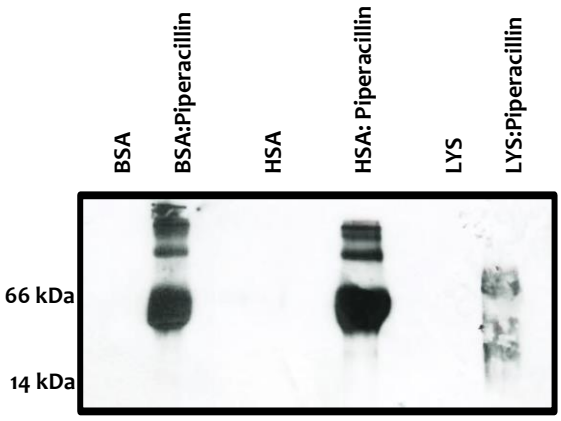


Figure 3.9. SDS-PAGE confirmation of Protein:piperacillin binding. Proteins and drug were mixed at a molar ratio of 1:50 then incubated for 96 hours at 37°C. Adducts were then precipitated with methanol, dried and dissolved in RPMI 1640. Samples (5 µg/ml) were run on SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was blocked with 2.5% milk in TST and incubated overnight at 4°C with a monoclonal mouse anti-penicillin antibody. After washing, the membrane was incubated with goat anti-mouse HRP-conjugated secondary antibody prior to ECL development with photographic film.

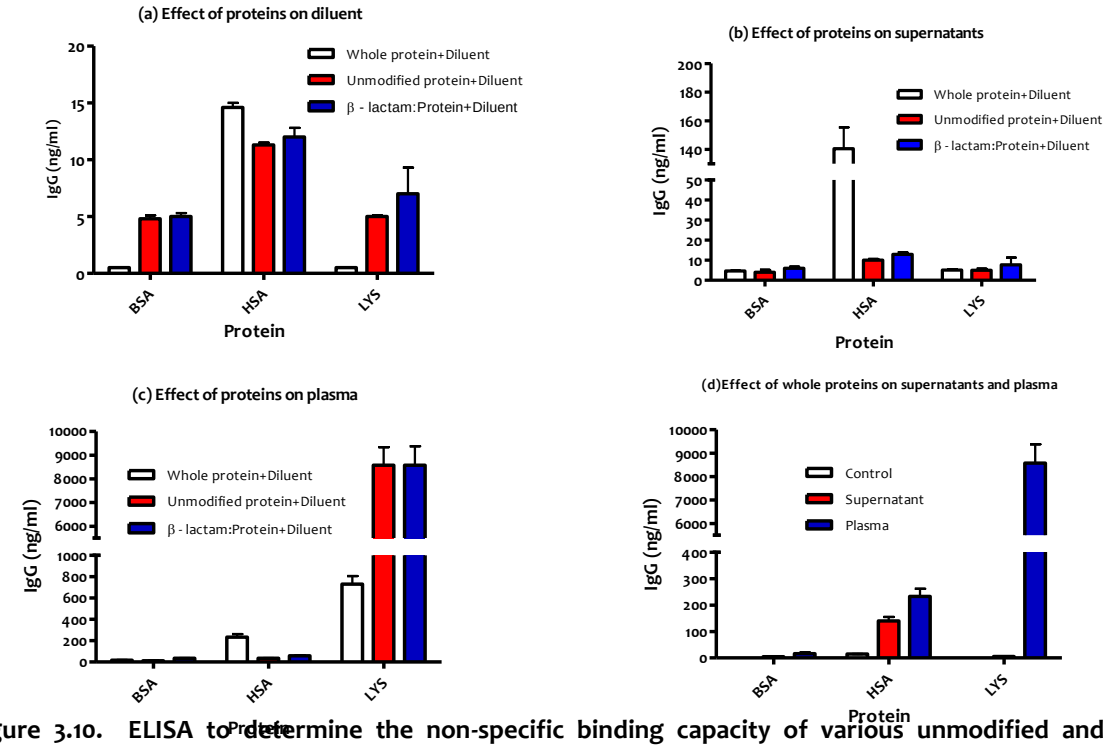


Figure 3.10. ELISA to determine the non-specific binding capacity of various unmodified and drug modified proteins. 20 µg/ml of unmodified bovine serum albumin (BSA), human serum albumin (HSA) and lysozyme were used to were used to coat flat bottomed high binding 96 well plates. After washing 100 µl of sample diluent (a) culture supernatants, (b) or plasma (c and d) was added were added and incubated for 1 hr. The presence of binding was detected by anti-IgG HRP conjugated antibody and TMB substrate. The reaction was stopped with 0.18M H_2SO_4.

3.2.9 *In vitro* characterization of BSA:Piperacillin binding in adducts generated for the detection of anti-drug antibodies

After the selection of the BSA:Piperacillin adduct for use after comparison of ELISA data, SDS-PAGE western blot analysis was carried out to confirm the time dependency of piperacillin adduct formation (figure 3.11). This was assessed using 5 μg/ml of each conjugate prepared at a ratio of 1:50 (protein:drug). The results show that a significant increase in the binding was observed at 96 hours when incubations at 96 and 24 hours were compared (fig 3.11). Mass spectrophotometric analysis of the 96 hour conjugate was carried out. The MS/MS spectrum of BSA lysine residue modification showed the characteristic internal peptide fragment ions m/z 160 and 143 (Figure 3.12). Piperacillin haptens were detected on 8 of the 13 lysine residues modified in HSA. The relative level of modification of the LYS residues are shown in figures 3.13 a and 3.13 b.

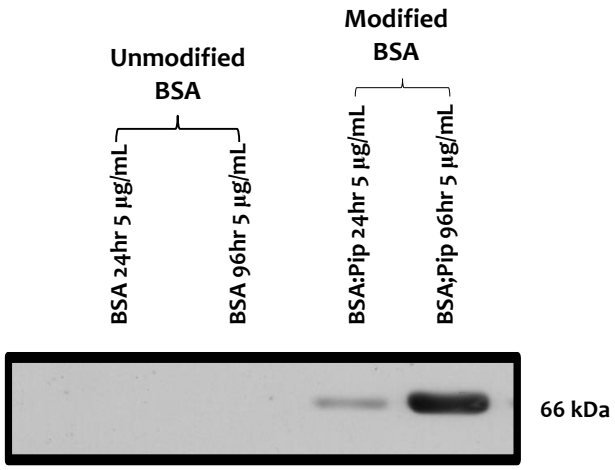


Figure 3.11 SDS-PAGE characterization of BSA:piperacillin adduct time dependent formation. BSA and piperacillin were combined at a molar ratio of 1:50 then incubated for 24 hours and 96 hours respectively at 37°C. Proteins and drug were mixed at a molar ratio of 1:50 then incubated for 96 hours at 37°C. Adducts were then precipitated with methanol, dried and dissolved in RPMI 1640. Samples (5 µg/ml) were run on SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was blocked with 2.5 % milk in TST and incubated overnight at 4°C with a monoclonal mouse anti-penicillin antibody. After washing, the membrane was incubated with goat anti-mouse HRP-conjugated secondary antibody prior to ECL development with photographic film.

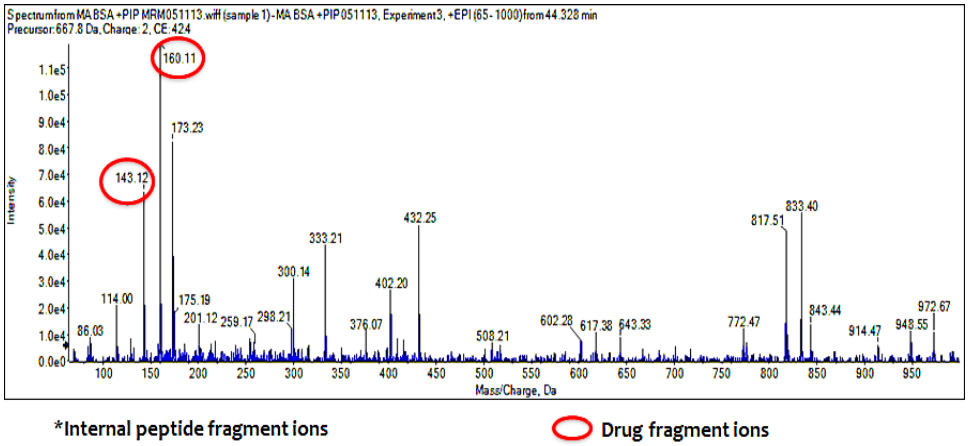


Figure 3.12 MRM spectral images of BSA lysine residue modification. Spectral images of MRM MS spectrometry analysis modification on LYS190 showing the presence of characteristic fragment ions at *m/z* 160 and 143.

	LYSINE	PEPTIDE
1.		ADEK*K
2.	211	ALK*AWSVAR
3.	131 20	DLGEEHFKGLVLIAFSQYLQ QCPFDEHVK
4.	4	DTHK*SEIAHR
5.	12	FK*DLGEEHFK
6.	136	FWGK*YLYEIAR
7.	535	HK*PK
8.	537	HKPK*ATEEQLK
9.	132	K*FWGK
10.	524	K*QTALVELLK
11.	431	SLGK*VGTR
12.	473	VTK*CCTESLVNR
13.	221	LSQK*FPK

* *Indicates site of modification*

Table 3.3 Incubation of piperacillin with BSA leads to the modification of 8 specific lysine residues. The binding of piperacillin to BSA leads to the modification of lysine residues in BSA. Mass spectrophotometric characterization of the formed adduct was carried out to determine the extent of residue modification. Adducts were denatured and treated enzymatically with trypsin. Desalting and enrichment of peptides was carried out prior to mass spectrometric analysis.

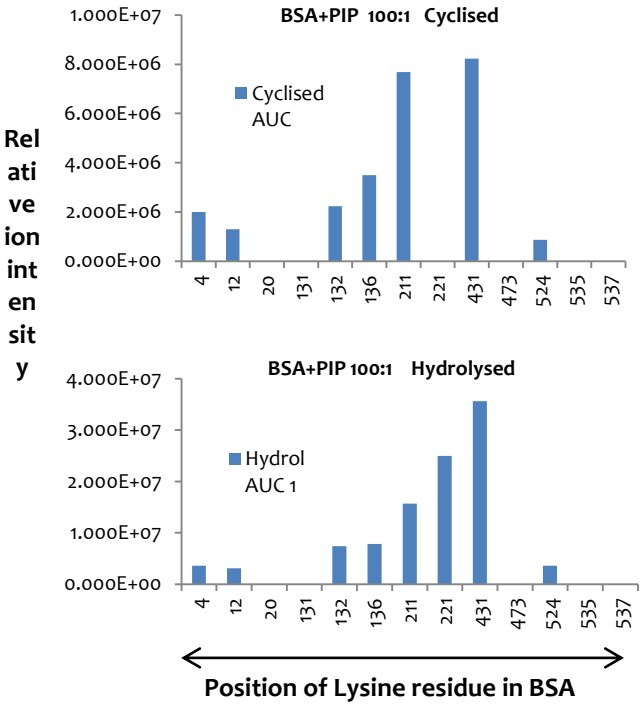


Figure 3.13

Lysine residues modified with cyclised and hydrolysed forms of the piperacillin hapten. Graphs showing the semi-quantitative analysis of covalent modifications at multiple sites on cyclised and hydrolysed piperacillin, at BSA:piperacillin molar ratio of 1:100 to enhance the modifications expressed by the protein. The abundance of modified peptides cannot be compared against each other because of their difference in ionisation efficiency. Cyclised and hydrolysed forms also ionise differently.

3.2.10 Detection of piperacillin-specific antibodies in hypersensitive patient plasma using piperacillin modified BSA as a drug antigen

Hapten inhibition assays were conducted using the plasma of piperacillin hypersensitive patient samples with CF. For the hapten inhibition plasma was pre-treated with an excess of the parent drug for 30 minutes. All hypersensitive patient samples contained anti-piperacillin antibodies, but at different levels ranging from < 250 ng/ml to >2000 ng/ml (figure 3.14 a - e). In each case addition of piperacillin for hapten inhibition reduced absorbance values to almost control levels.

To confirm the specificity of the antidrug antibodies p-phenylenediamine and isoniazid conjugates were prepared according to methods used by Jenkinson et al. and Meng et al. (Jenkinson, Jenkins et al. 2010, Meng, Maggs et al. 2014) respectively. Addition of patient plasma alone or with the addition of the anti-piperacillin antibody inhibitor did not produce an increase in absorbance values indicating the IgG did not bind to these alternative drug/chemical antigens. As expected, addition of piperacillin for hapten inhibition had no effect (figure 3.15 a - b).

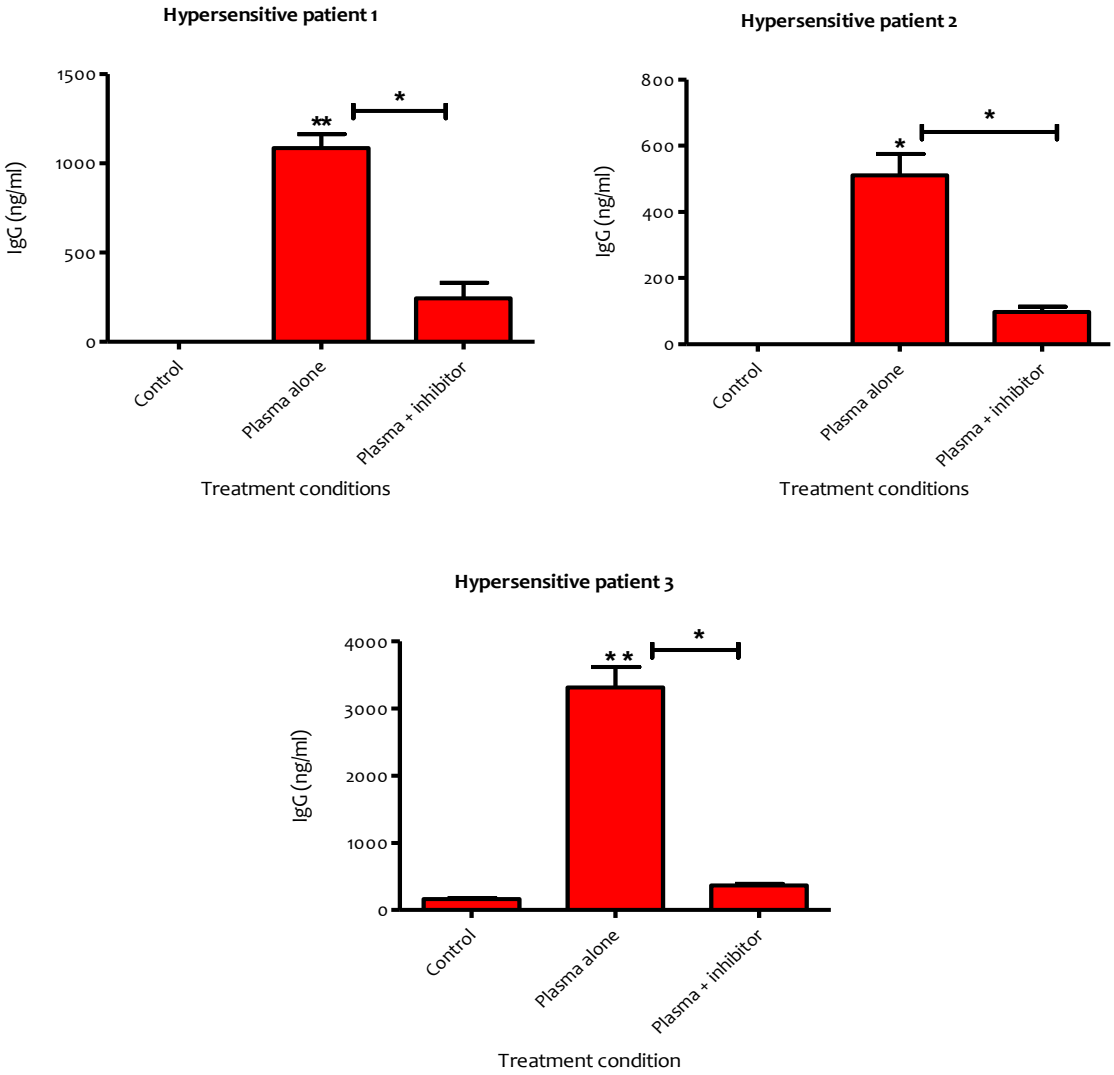


Figure 3.14. Anti-piperacillin-specific IgG ELISA with hypersensitive patient plasma. Flat bottomed 96 well plates were pre-coated with 20 µg BSA:piperacillin adduct employed as an antigen. After washing 100 µl of hypersensitive patient plasma with and without prior incubation with the hapten, was added to wells and an ELISA carried out. Absorbance was read with an automated plate reader (Dynatech MR600). Data were analysed by the Students T test to compare the difference between means. $p \leq 0.05$ considered as significant. (* denotes $p < 0.05$; ** denotes $p < 0.01$).

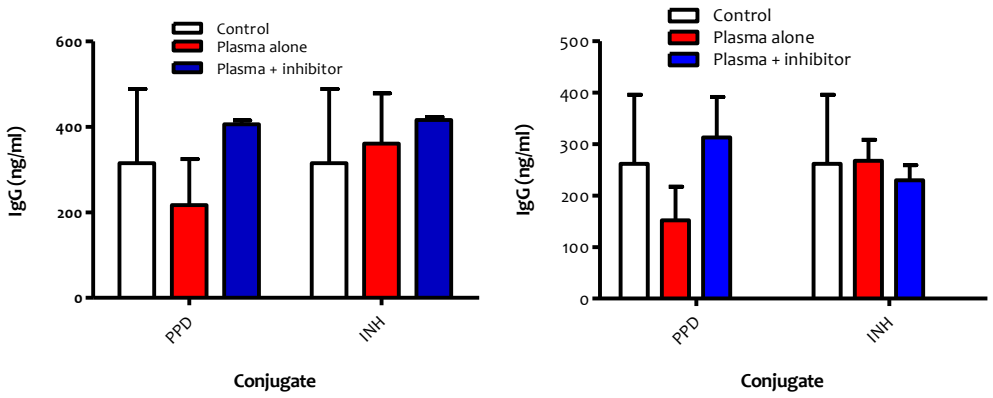


Figure 3.15 Confirmation of the specificity of anti-drug antibodies to piperacillin haptens using p-phenylenediamine (PPD) and isoniazid (INH) conjugates. Flat bottomed 96 well plates were pre-coated with 20 µg HSA:PPD and HSA:INH piperacillin adduct. After washing 100 µl of hypersensitive patient plasma with and without prior incubation with the hapten, was added to wells and an ELISA carried out. Absorbance was read with an automated plate reader (Dynatech MR600). Data were analysed by the Students T test. No significant differences ($p \leq 0.05$) were observed between the data.

3.2.11 Generation and characterisation of IgG secreting B-cell lines, from piperacillin hypersensitive patients

B cells from piperacillin naïve, and hypersensitive patients were transformed with Epstein-Barr virus. The immortalization was carried out with and without the incorporation of cpg-dna according to the methods described in section 2.7(i) and section 2.7 (ii). The different populations were initially analysed for total IgG secretion using an ELISpot assay. Immortalised B cell lines from naïve volunteers treated with piperacillin and cpg-dna did not produce any significant spots even when higher numbers of cells were used in the ELISpot assay. B-cell lines generated from hypersensitive patients and treated with piperacillin and cpg-dna produced spots indicative of IgG secretion with an increased frequency of IgG producing cells as the number of seeded cells per well was increased (figure 3.16).

Results from flow cytometry analysis of immortalised B cells indicated the substantial expression of $CD19^{+}27^{+}$ surface marker expression on the majority of lines, with values ranging from 11.4% to 57.69% of the total visible population of cells. This expression varied widely from one line to the other (figure 3.17 a). In fact, some cell lines were only positive for $CD19^{+}$ (figure 3.17 b). Furthermore several lines did not express either CD19+ or CD27+ (figure 3.17 c).

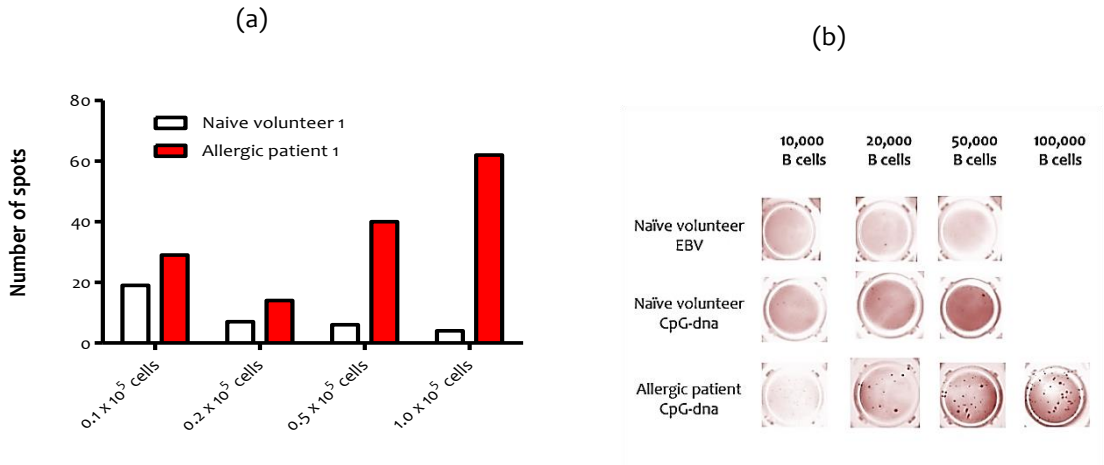


Figure 3.16 Analysis of immortalised cell lines for total IgG secretion using an ELISpot assay. The ELISpot plates were pre-coated with anti-human IgG according to the manufacturer's instructions and incubated overnight at 4°C. Immortalised B cells were harvested and four cell concentrations were transferred to each well and incubated for 48 hours. ELISpot plates were developed according to the manufacturer's instructions, and the wells left to air dry. Data were analysed and images captured using an AID ELISpot machine. (a) Spot counts (b) ELISpot pictures.

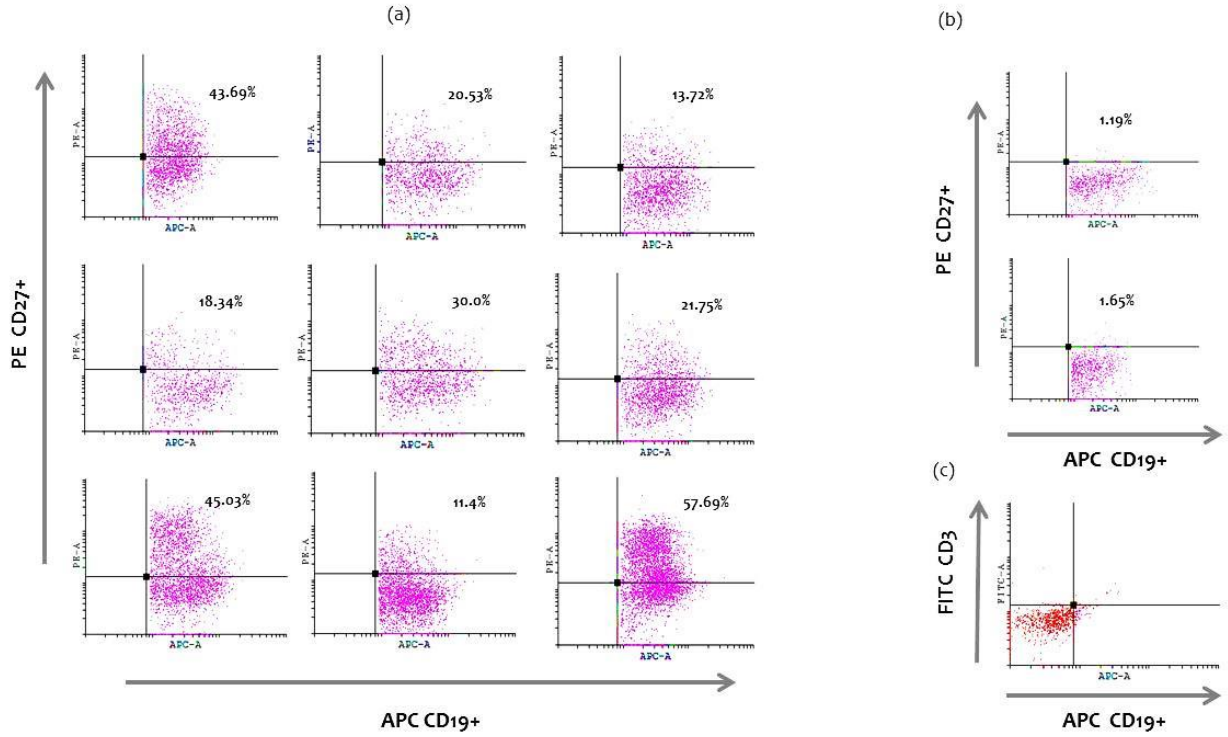


Figure 3.17. CD19+27+ expression on B-cell lines. Twelve cell lines from a hypersensitive patient were stained with CD3-FITC, CD19-APC and CD27-PE fluorochrome antibodies and analysed by flow cytometry to determine the phenotype of the immortalized cells. Dot plots show (a) $CD19^{+}27^{+}$ phenotype (b) $CD19^{+}27^{-}$ phenotype and (c) non-CD19 phenotype.

3.2.12 Detection of anti-drug antibodies in B-cell lines

B cell lines were generated from the 3 piperacillin hypersensitive patients. After immortalization and TLR9 stimulation 48 B cell lines were produced from patient 1, 140 lines from patient 2 and 160 lines from patient 3. From the total number of lines generated 8, 12 and 15 lines respectively were found to secrete IgG after testing using an IgG ELISA. Thus the percentage IgG secreting lines generated from each patient were 16%, 8.5% and 9.3%, respectively (Table 3.4). The introduction of Th cytokines to emulate a T cell dependent immortalization of EBVS saw an enhancement in the efficiency of IgG secreting B-cell line generation to 99%.

Table 3.3. Immortalization of IgG secreting B-cell lines from hypersensitive patients. Immortalized B-cell lines were generated from 3 hypersensitive patients utilizing T-cell independent and T-cell dependent methods incorporating specific Th cell cytokines. Supernatants from these cell lines were tested for IgG secretion by ELISA and the efficiency of generation calculated. Tests for anti-piperacillin-specific IgG in these cell lines proved negative.

	Total number of cell lines generated.	Total number of cell lines secreting IgG	Efficiency of generation (%)	Number of cell lines secreting piperacillin-specific antibody
Hypersensitive patient 1	48	8	16%	Nil
Hypersensitive patient 2	140	12	8.5%	Nil
Hypersensitive patient 3	160	15	9.3%	Nil
Hypersensitive patient 3 (T cell dependent)	120	120 (Majority at levels < 500 ng/ml)	99.2%	Nil

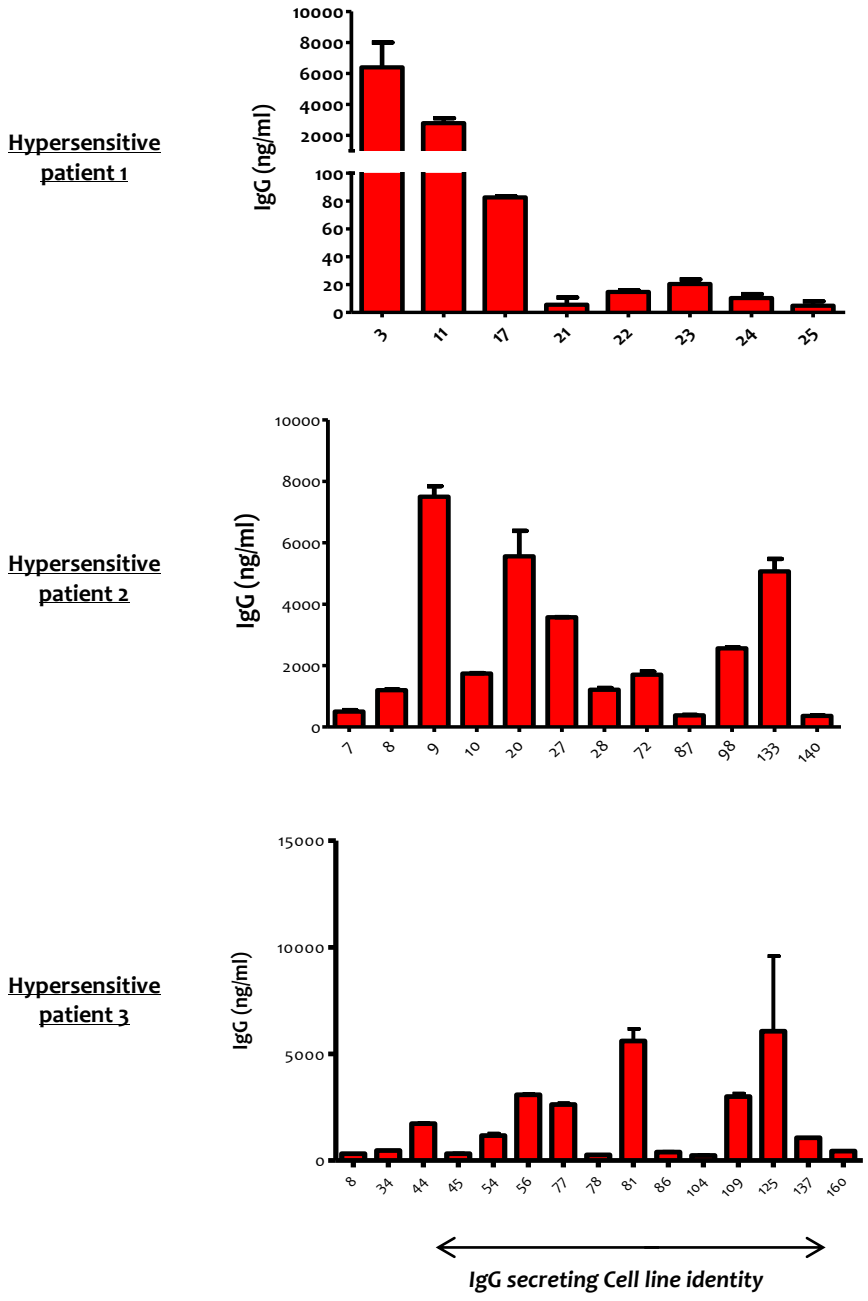


Figure 3.19. Secretory profile of B-cell lines producing IgG. B cells from 3 hypersensitive patients were immortalised by Epstein-Barr virus transformation in a T-cell independent manner. Supernatants were collected and assessed for IgG secretion. Flat bottomed high binding 96 well plates were pre-coated with 10 µl/ml anti-IgG overnight and 100 µl of plasma was added to the wells in duplicate. Colour development was performed with HRP and TMB substrate and stopped with 0.18M H_2SO_4.

Utilizing supernatants from the IgG producing lines and employing a hapten inhibition ELISA, lines were tested for the presence of antibodies specific for piperacillin. None of the lines generated from the patients were found to secrete IgG with specificity for piperacillin.

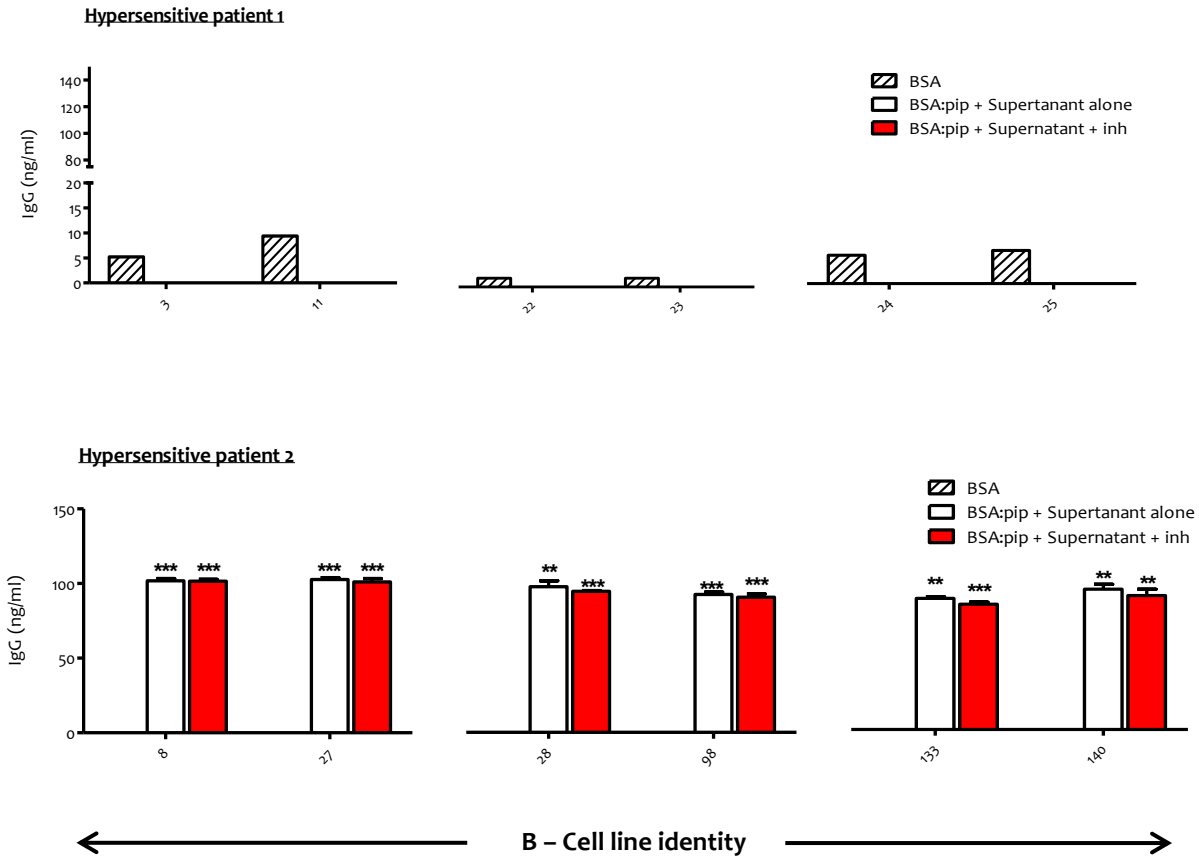


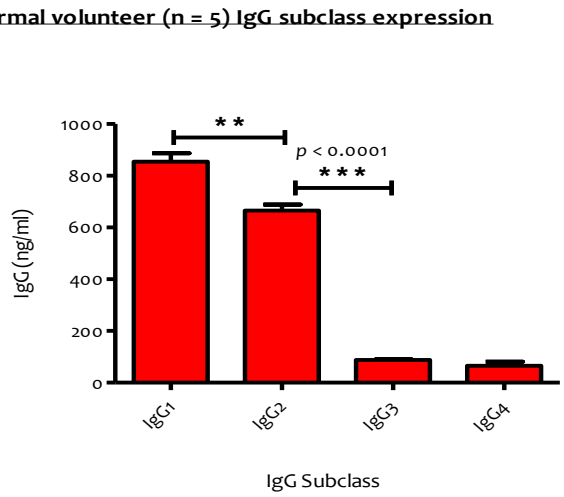
Figure 3.20 Anti-piperacillin antibody is not detectable in hypersensitive patient B-cell lines. Secretion of anti-piperacillin antibody by 6 B cell lines from piperacillin hypersensitive patient 1 and 8 B cell lines from hypersensitive patient 2 was assessed by ELISA. High binding U-bottom 96 well plates were pre-coated with 20 µg/ml BSA:piperacillin adducts and incubated overnight at 4°C. After washing 100 µl Cell culture supernatants diluted at a ratio of 1:10 (Supernatant:diluent) was added per well and incubated for 1 hr. The presence of drug specific antibodies was detected by anti-IgG HRP conjugated antibody and TMB substrate. The reaction was stopped with 0.18M H_2SO_4 and absorbance measured at 470nm using a spectrophotometer. The data were analysed by Students T test with $p<0.05$ considered significant (* denotes $p<0.05$;** denotes $p < 0.01$).

3.2.13 IgG sub-class analysis in patient plasma and B-cell line supernatants

IgG sub-classes were assessed in plasma of naïve volunteers and, plasma and B cell line supernatants from piperacillin hypersensitive patients. IgG subclass analysis of naïve volunteers (n=5) produced a subclass profile with antibody levels in the order of IgG1 > IgG2 > IgG3 > IgG4 (figure 3.21 a). This is in agreement with previously established IgG subclass values in human sera (Morell, Skvaril et al. 1971, Oxelius 1978, Madassery, Kwon et al. 1988, Schauer, Stemberg et al. 2003).

The subclass profile of IgG antibodies secreted from B-cell lines was assessed to determine if there was any variation from the norm. IgG subclass expression from selected antibody secreting lines generated from piperacillin hypersensitive patient 1 showed a profile similar to that observed in the plasma of control group of patients (Figure 3.21).

(a)



(b)

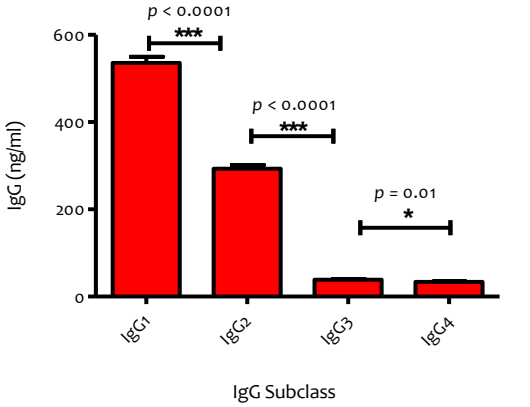


Figure 3.21 IgG sub-class secretory profiles of normal volunteers. (a) Plasma from 5 naïve volunteers was analysed and (b) 5 B cell lines form hypersensitive patient 3 were analysed. Flat bottom high binding 96 well plates were pre-coated with 20 µg/ml anti-IgG antibody, and incubated overnight at 4°C. After washing 100 µl plasma was added to each well and incubated for 1 hour. After washing subclass specific antibodies were added. Colour development by reaction of HRP and TMB substrate was stopped with 0.18M H_2SO_4. Absorbance was read with an automated plate reader (Dynatech MR600) with the reference wavelength set at 630 nm. The data were analysed by Students T test with p<0.05 considered significant (* denotes $p < 0.05$;** denotes $p < 0.01$).

BSA:piperacillin adducts generated using methanol precipitation as described earlier, were used to detect the presence or not of measureable amounts of IgG1, IgG2, IgG3, and IgG4 subclasses that were specific to piperacillin. Using plasma samples (n=3) from

hypersensitive patients, mass values for subclass-specific anti-piperacillin IgG in hypersensitive patient plasma samples were designated. Subclass-specific anti-piperacillin IgG expression showed varied patterns of IgG1 and IgG2 antibody expression in the three patient samples. Patient 1 showed a profile consistent with that observed previously with naïve volunteers and tolerant patients in figure 3.21. Patient 2 expressed IgG1 and IgG2 at 19.58% and 27.8% of the total piperacillin-specific IgG, while patient 3 expressed IgG1 and IgG2 at 23.4% and 20.4% of total piperacillin-specific IgG (fig 3.22).

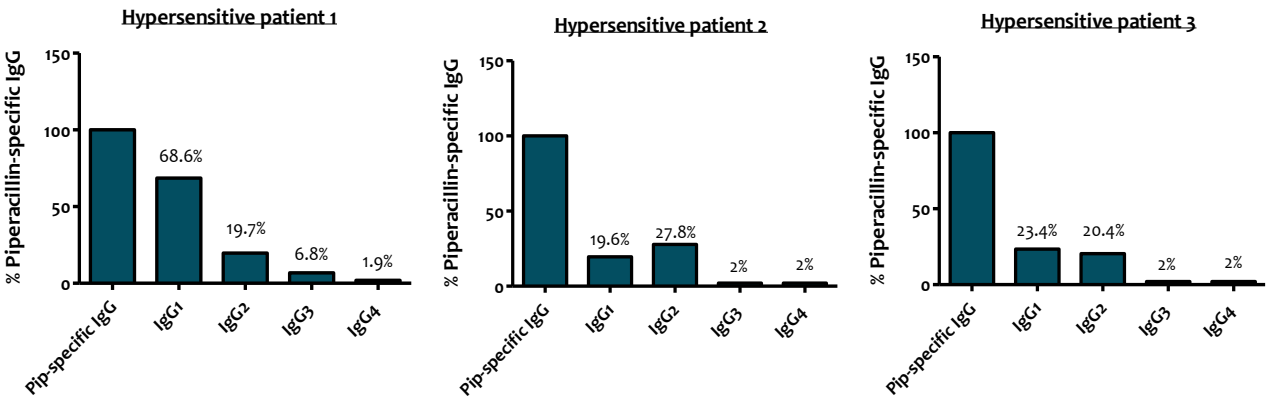


Figure 3.22 IgG sub-class specific responses to piperacillin stimulation. The amount of different IgG subclasses in the plasma from 3 piperacillin hypersensitive patients was analysed by ELISA. Wells of the high binding 96 well plates were pre-coated with 20 µg/ml BSA:piperacillin adduct and incubated overnight at 4°C. 100 µl of hypersensitive patient plasma was added to wells in duplicate. This was followed by the addition of IgG1, IgG2, IgG3, and IgG4 subclass specific antibodies. 100 µI of streptavidin (diluted 1:1000 for IgG1 and 1:10,000 for IgG2, IgG3 and IgG4) were added and the assay developed with TMB. The colour change was then read with automated plate reader (Dynatech MR600) with the reference wavelength set at 630 nm. The data were analysed by Students T test with $p < 0.05$ considered significant (* denotes $p < 0.05$;** denotes $p < 0.01$; *** denotes $p < 0.001$).

3.3 Discussion

The cellular and humoral components of the immune system have been described in great detail. Characterization of the humoral response to infectious organisms and in autoimmune disorders have been defined in mice (Teixeira, Marques et al. 2005, Yang, Rui et al. 2013) and humans (Wahlgren, Berzins et al. 1983, Grammer and Lipsky 2003, den Reijer, Lemmens-den Toom et al. 2013).

B cells play a critical role in humoral responses. The protective function of IL-10 secreting B regulatory cells (Bregs) has been demonstrated in experimental models of arthritis (Yanaba, Bouaziz et al. 2008). In contrast the pathogenic role of B cells has been described in models of type 1 diabetes. Non-obese diabetic (NOD) mice have the partial or total loss of B cells, which correlates directly with the development of diabetes (Xiu, Wong et al. 2008, Marino, Villanueva et al. 2009), with a reduction in the number of circulating B cells leading to a reduction in the development of insulitis. Researchers have utilised the advances in B cell biology to enable the identification of specific immunoglobulins (Christie, Coleman et al. 1988), the development of methods for the identification of immunoglobulin subclasses (Lefevre, Carr et al. 2009), and the effector and regulatory roles they perform in the immune process (Lund 2008) to enable an understanding of the contribution of B-cells to self-recognition, specificity and immunologic memory.

Characterization of the cellular immune response in delayed-type drug hypersensitivity has been thoroughly explored (Naisbitt, Farrell et al. 2003, Naisbitt, Farrell et al. 2005, Whitaker, Meng et al. 2011, El-Ghaiesh, Monshi et al. 2012), but the corresponding

humoral responses have been knowingly or otherwise neglected. Thus, in this chapter the objective was to evaluate whether existing methods could be modified or new cell culture methods developed to detect drug-specific B cell responses. Piperacillin hypersensitive patients with cystic fibrosis were selected as the study cohort for 3 reasons. First hypersensitive, tolerant and drug-naïve patients were willing to participate; second, the nature of the cellular response to piperacillin has been fully defined; and third, the binding of piperacillin to protein in patients plasma has been characterized; hence methods are available to develop drug-protein adducts as antigens. The drug-specific activation of B-cells, the development of antibody producing B cell lines and the characterization of the IgG antibodies generated by these cells, including their subclasses was determined. Further analysis was performed to detect piperacillin-specific antibodies in cell supernatants and plasma.

In order to achieve these goals PBMCs were isolated from piperacillin naïve volunteers, piperacillin tolerant and hypersensitive patients with cystic fibrosis. The analysis of the proliferation of PBMCs to piperacillin was performed using the lymphocyte transformation test (LTT), which is a simple assay for diagnosis, to verify sensitization to the drug. T-lymphocytes from hypersensitive patients, but not tolerant controls or naïve donors were stimulated to proliferate in the presence of the drug. The peak concentration for T-cell activation was 1 mM. Proliferative responses started to taper off at 4 mM, in most cases. Thus, the same concentrations of piperacillin were used to detect B-cell proliferative responses in each patient group. The detection of B-cell responses and specific IgG secretion is important as antibodies play a pathogenic or regulatory role in the development of drug hypersensitivity.

To detect the B cell responses to either piperacillin or cpg-dna, PBMC were cultured with antigen or mitogen for 5 days prior to the detection of specific B cell markers by flow cytometry and/or IgG secretory profiles by ELISpot. Cpg-dna was selected as a positive control because of its propensity to activate immature and mature B cells via TLR9 stimulation (Azulay-Debby, Edry et al. 2007) leading to cell proliferation and antibody production (He, Qiao et al. 2004).

Phenotypic assessment of the different treatment groups showed that while they all expressed CD19 indicative of B-cells, only the hypersensitive patients showed an increase in expression of the activation marker CD27+ in response to *in vitro* piperacillin stimulation. This increase in peripheral levels of memory B cells is indicative of an existing reservoir of piperacillin-specific B cells generated during prior piperacillin exposure, likely around the time of hypersensitivity reaction. This coincides with previous studies confirming the increase in frequencies of memory B cell subsets in a population of *Schistosoma Haematobium* infected patients (Labuda, Ateba-Ngoa et al. 2013). The tolerant patients B-cells did not display an increase in CD27 expression after exposure to the drug. Similarly B-cells from naïve donors were not activated with piperacillin.

Stimulation of hypersensitive patient PBMC also led to an increase in the secretion of IgG, detection of which was performed using an IgG ELISpot assay, utilizing PBMCs harvested from a 5 day culture. In contrast no difference in IgG secretion was observed with PBMC of naïve volunteers and tolerant patients after drug treatment. Cpg-dna, which has the capacity to initiate the activation of naïve B cells and the release of immunoglobulins M and G (Huggins, Pellegrin et al. 2007) was used as a positive control. High levels of IgG secretion was consistently observed in each patient group. Collectively, these data

indicate that piperacillin-specific B-cells are present only in hypersensitive patients. Later chapters expand on these data by assessing a larger patient cohort, defining the specificity of the antibody response and the role of B-cells in regulating piperacillin-specific T-cell responses.

The detection of antibodies specific to penicillin in patient plasma was first described several decades ago (Rammelkamp and Keefer 1943, Kern and Wimberley 1953). Penicillin was also shown to bind proteins such as HSA (Tompsett, Shultz et al. 1947) and the pharmacological implication with respect to penicillin activity also explored (Ley, Harris et al. 1958). Nowadays research has been able to further confirm the presence of antibodies specific to β-lactams. The binding of these drug haptens to proteins, their subsequent peptide synthesis and presentation by MHC to T cells have now been identified as key precursors for the onset of reaction. However significant questions still remain unanswered concerning the role B-cells and their secretory products play in the inhibition and regulation of delayed hypersensitivity.

The Enzyme Linked Immunosorbent Assay (ELISA) has proved useful in the detection/assessment of antibody responses in a variety of cellular compartments (Bishai and Galli 1978, McNeil, Simpson et al. 1990). An anti-IgG antibody was used for the detection of total IgG secretion in B-cell line supernatants and in piperacillin hypersensitive patient plasma. IgG ELISA analysis of supernatants from PBMC cultured with piperacillin showed low level detection of IgG secretion in response to piperacillin in each patient group. Thus, it seems likely that the number of memory B cells activated with piperacillin in the *in vitro* culture was insufficient to generate IgG levels required for detection by ELISA. This necessitated the generation of IgG secreting B cell lines from

hypersensitive patients and/or the use of plasma samples wherein we were able to assess the different subclasses and attempt to confirm the presence of IgG antibodies specific to piperacillin.

Substantial levels of IgG antibody was detected in plasma suggesting that quantifiable amounts of IgG subclasses could possibly be detected. Piperacillin-HSA adducts were generated and employed as antigens in hapten-inhibition assay for unambiguous determination of antibody specific to the piperacillin hapten. Unmodified HSA produced a high background apparently binding IgG itself; hence its further use was discontinued. The fact that piperacillin binds specifically to Lys residues (Whitaker, Meng et al. 2011) meant that an appropriate replacement would need to be rich in lysine. Thus, HSA was compared with BSA and lysozyme, two Lys rich proteins that have been used previously to form conjugates for the assessment of drug specific responses (Coleman, Yeung et al. 1986, Haas, Moolenaar et al. 2002, Yeh, Lee et al. 2008, Haselberg, Harmsen et al. 2011, Nguyen, Bobst et al. 2013). Different parameters of the assay including diluents used, PBMC culture supernatants, and plasma were assessed.

Piperacillin-protein adducts with modifications arising at multiple lysine residues was detected with all proteins. Low backgrounds in the ELISA was observed when BSA was compared to that of HSA and lysozyme suggesting that BSA is a suitable protein for the generation of piperacillin antigens to detect specific IgG.

Previous studies aimed at establishing a pattern for the levels of specific IgG in tolerant and hypersensitive patients have produced diverse results ranging from no observable significant changes between the groups (Sletten, Halvorsen et al. 2006), to elevated IgG

levels in clinically reactive patients when compared to tolerant individuals (Lopes de Oliveira, Aderhold et al. 2013).

Comparison of the IgG subclass profiles in plasma between naïve volunteers, and patient samples did not manifest any differences in expression. The generally described ratios of IgG1>IgG2>IgG3>IgG4 was shown in hypersensitive patient and naive volunteer plasma as well as IgG secreting B-cell lines. Other investigators have previously identified an elevation in serum levels of IgG4 though it has been explained as a by-product of the general increase in total IgG and not a feature of any clinical condition (Shryock, Molle et al. 1986, Moss 1987, Hodson, Morris et al. 1988). Using the piperacillin BSA antigen, a hapten inhibition ELISA was used to detect the presence of antidrug antibodies specific to piperacillin in patient plasma samples. Antidrug antibodies specific to piperacillin were detected in plasma from hypersensitive patients. This expression was not limited to the reactive stage of drug treatment but in some instances was shown to be present several months or even years after the termination of therapy. This is not surprising considering the fact that most of the patients have been exposed to the drug previously, and would be expected to develop an immunologic memory with antibodies remaining quiescent until re-exposure. An attempt to ascribe the specificity to a specific IgG subclass using a modified ELISA shows substantial interindividual variability (figure 3.22) the sample size used (n =3) was small thus a larger sample size would have to be employed before a definite conclusion can be reached.

The phenotypic characterization of B cell lines and analysis of their IgG secretory profiles was carried out primarily to assess their potential to produce anti-drug antibodies specific to piperacillin. However, if successful these lines would also be used in T-cell

assays as antigen presenting cells to assess whether piperacillin-specific IgG has the ability to regulate drug-specific T-cell responses.

In vitro infection of B-cells with EBV under controlled conditions resulted in immortalization. The EBV induced proliferation and differentiation with eventual fixation in the lymphoblast stage are thought to be the pathways by which indefinite differentiation leading to immortalization occurs (Thorley-Lawson and Mann 1985). The virus binds to the C3d receptor (complement receptor) and cells subsequently express viral genes amongst which include the EBV nuclear antigens (EBNAs), latent membrane proteins (LMP) and RNA species called EBV-encoded RNA's (EBERs) (Fingeroth, Weis et al. 1984). The EBNAs have been implicated in the reduced dependence on serum factor in mice, enhanced expression of CD23 (B-cell activation antigen) and CD21 and the development of lymphocytic lymphoma and leukaemia in transgenic mice B-cells (Dambaugh, Wang et al. 1986, Wang, Gregory et al. 1987, Wilson and Levine 1992).

Long lived IgG secreting B cells were generated using methods modified from that employed by Lanzavecchia et al (Lanzavecchia, Corti et al. 2007). CD19 expressing cells were cultured in the presence of Epstein-Barr virus (EBV), irradiated PBMCs and CpG-dna which activates B-cell via TLR9 stimulation (see chapter 2, figure 2.3). IgG secreting immortalized B-cells from hypersensitive patients were identified by ELISA and ELISpot assays. They were shown to express a pure $CD19^{+}CD27^{+}$ phenotype consistent with the memory B cell phenotype exhibited by normal B cells. Though there was a sustainable production of IgG in many immortalized lines the inability to detect anti-drug antibodies in these lines should also be noted. The addition of IL-4 and IL-5 during the immortalization as exogenous sources of Th cytokines produced an increased efficiency

in the generation of IgG secreting B cells. However, these lines with the ability to produce antibodies still did not secrete piperacillin-specific IgG (Schultz and Coffman 1991, MacLennan 1994, Manis, Tian et al. 2002).

Future experiments should be directed towards modification of methods utilized for the generation of B-cell lines for the purpose of producing antigen specific antibody. Efforts could also be made to assess isotype switching, somatic hypermutation, and class switch recombination to fully understand the way molecular processes are affected. Collectively these data show the availability of *in vitro* experimental methods to define B cell activation in disease states in general, and drug hypersensitivity in particular, and points us in a new direction in the characterization of the humoral response in hypersensitive patients.

CHAPTER 4

CHARACTERIZATION OF ANTIDRUG ANTIBODIES IN PIPERACILLIN HYPERSENSITIVE PATIENTS, AND ASSESSMENT OF β-LACTAM CROSS-REACTIVITY.

Table of contents

Figures

Tables

4.0 Introduction

The β-lactams are a group of drugs which include a β-lactam ring as a major component of the structure. These drugs are either used alone or in combination with other chemical agents referred to as β-lactamase inhibitors which serve to conserve activity and extend their spectrum (Lee, Yuen et al. 2001). Classically, β-lactams inhibit bacterial cell growth leading to death and lysis. These results are directly linked to the inhibition of enzymes responsible for peptidoglycan synthesis responsible for the maintenance of bacterial cell wall integrity. β-lactams bind covalently to multiple proteins located in the bacterial cell membrane collectively referred to as penicillin binding proteins (PBPs). Previously β-lactams were thought to bind through the formation of an ester; however it has now clearly been shown that the β-lactam is attached via the penicilloyl moiety. (Waxman and Strominger 1983)

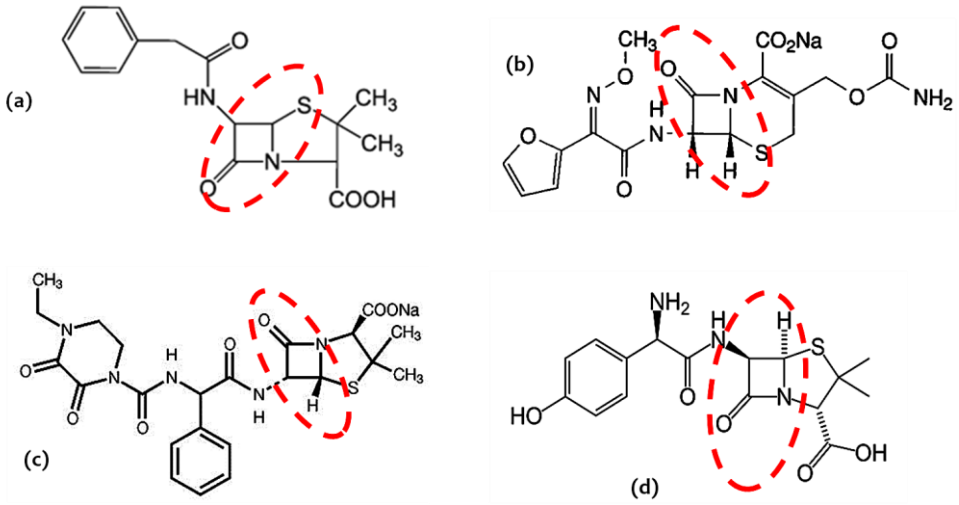


Figure 4.1. β-lactam antibiotic structures. Structure of 4 β-lactam antibiotics: (a) penicillin G (b) cefuroxime a cephalosporin (c) piperacillin and (d) amoxicillin showing the core β-lactam nucleus (encircled).

β-lactams have been implicated in the initiation of a plethora of adverse drug reactions which are for the most part cutaneous in nature. These reactions have been observed

with structurally related drugs. Thus, there is concern of cross-reactivity with structurally similar and diverse drugs that contain the β-lactam ring, including amoxicillin, piperacillin and cephalosporins (Figure 4.1) (Crieco 1967, Tsuchiya, Shiho et al. 1979, James and Gurk-Turner 2001, Arndt and Garratty 2002, Terico and Gallagher 2014). Clinical manifestations vary in severity ranging from maculopapular rashes to erythema multiforme, Drug reactions with eosinophilia and systemic symptoms (DRESS) and Stevens-Johnson syndrome and Toxic epidermal necrolysis (SJS-TEN). The mechanisms that lead to tissue injury generally involve activation of the host immune system. The basic concepts have been elaborated upon in chapter 1.

Piperacillin is a β-lactam antibiotic used to combat respiratory tract infections in patients with CF. Cutaneous hypersensitivity reactions occur at a higher frequency in patients with CF when compared to the general population (Whitaker, Naisbitt et al. 2012) with an incidence of up to 30 % reported in some studies. Thus, such reactions represent an ideal model to study the role of drug-specific T and B lymphocytes in the disease pathogenesis (Whitaker, Naisbitt et al. 2012).

The role played by proteins in the initiation of β-lactam hypersensitivity reactions is becoming clearer by the day. Substantial evidence now exists to show that the binding of a drug hapten to a protein as originally proposed by Landsteiner and Jacobs is a prominent mechanism for immunogenicity and hypersensitivity (Chipinda, Hettick et al. 2011). A number of mechanisms for the immunogenicity of these haptenated proteins have been proposed. They include but are not restricted to their multivalent nature, differences in the affinities, avidities, and precursor frequencies of antigen-specific cells, and the likely contribution of protein denaturation due to haptenation (Elahi, Wright et

al. 2004). While the mechanism of immunogenicity of haptens remains ill defined, we know for a fact that modification of skin proteins influence the relative skin sensitisation by cinnamic compounds (Basketter 1992, Cheung, Hotchkiss et al. 2003). The formation of benzylpenicillin-protein conjugates, and further mass spectrometric characterization to reveal the diastereoisomeric nature of the benzypenicilloyl adducts has recently been described (Meng, Jenkins et al. 2011). Most recently, piperacillin has been shown to modify lysine residues present in human serum albumin *in vitro* and in drug-exposed patients (Whitaker, Meng et al. 2011, El-Ghaiesh, Monshi et al. 2012). Importantly four hapten structures are found; a primary cyclised and hydrolysed hapten structure and desethyl metabolites of both structures (see figure 4.2c). A synthetic piperacillin-HSA adduct was found to activate T-cells isolated from hypersensitive patients (El-Ghaiesh, Monshi et al. 2012). However to date it is not known whether one or all adduct structures activate the immune response.

Adducts have also been detected in patients exposed to other classes of drugs. For example diclofenac, abacavir and isoniazid all modify HSA cysteine, lysine, and/or histidine groups in exposed patients (Hammond, Meng et al. 2014, Meng, Lawrenson et al. 2014). In each of these examples adduct formation is dependent on drug metabolism and liberation of a chemically reactive intermediate. In contrast to piperacillin the immunogenicity of such adducts has not been studied. In addition, abacavir binds non-covalently to the floor of the peptide binding groove of HLA-B*5701 thereby altering the repertoire of self-peptides that load on the molecule to be presented to the immune system as shown by Illing, Vivian et al. (as stated in chapter 1) a mechanism suggested to

predispose individuals to abacavir hypersensitivity reactions. Thus the role of abacavir protein adducts in the hypersensitivity reaction has not been defined.

Drugs and metabolites have previously been incubated with proteins or peptides to determine their reactivity, and also to detect the presence of circulating antibodies (Coleman, Yeung et al. 1986, Annunziata, Kohn et al. 2013). Efforts have been made to understand the role of hapten density on the intensity of the immune response, as shown in the study carried out by Hu and others (Hu, Huang et al. 2012), and also on the antibody repertoire/spectrum induced by conjugates at different densities (Li, Rodriguez et al. 2010). The main downside of these studies has been the failure to elucidate the role the various lysine residue modifications play in the detection of anti-drug antibodies and subsequent intensity of the immune response and also the possibility of functioning as reactive epitopes.

The presence of IgE-mediated penicillin hypersensitivity generally predisposes an individual to similar reactions in response to exposure to other β-lactams. This cross reactivity, usually occurs between β-lactams and β-lactam classes (Preston, Briceland et al. 1994, James and Gurk-Turner 2001). However different rates/incidences of hypersensitivity are observed in different patient groups. For non-IgE mediated reactions, the side chain of the β-lactam antibiotic is thought to be involved in antigen specificity; however our knowledge of IgG drug-protein conjugate binding is limited (Robinson, Hameed et al. 2002).

Thus the aims of this chapter were to;

I. Characterize piperacillin (Pip) covalent binding to protein.

II. Quantify the time and concentration dependency of piperacillin covalent binding to protein and the antidrug antibody response.

III. Assess antibody cross reactivity across a spectrum of β-lactams.

4.1 Methods

See material and method sections on Gel electrophoresis for confirmation of adduct formation (2.3.2), SDS-PAGE western blot (2.3.2.1), mass spectrometric analysis (2.3.3), trypsin digests for mass spectrometric analysis (2.3.3.1), matrix assisted laser desorption ionisation (MALDI) (2.3.3.2), multiple reaction monitoring characterization of β-lactam albumin binding (2.3.3.3). Modification of ELISA for the detection of anti-drug antibody levels in tolerant and hypersensitive patient plasma samples to assess hapten density assay cross reactivity is discussed in section 2.2.3.2.

4.2 Results

4.2.1 Confirmation of drug-protein binding in Piperacillin-BSA conjugate

Cell culture methods to detect the B-cell response in hypersensitive patients were developed in chapter 3. Identification of piperacillin-specific IgG using Piperacillin-BSA adducts prepared as antigens was carried out. A piperacillin-BSA adduct was characterized using western blotting to confirm piperacillin-protein binding, and mass spectrophotometric analysis was performed to define the extent of lysine residue modification. Figure 4.2 provides a summary of piperacillin-BSA binding and characterization carried out in the previous chapter as a preamble to further studies carried out in this chapter. SDS-PAGE western blot was used to characterize the time-dependent piperacillin-BSA binding at 24 and 96 hours. A higher degree of Piperacillin-BSA adduction was observed at 96 h compared to 24 h (Figure 4.2a). The 96 h time point was picked for subsequent studies on adduct formation. The extent of lysine residue modifications by cyclised and hydrolysed piperacillin covalently bound to BSA at 1:100 molar ratio of BSA-piperacillin was also described (Figure 4.2b). The identification of binding and detection of modified residues set the stage for further characterization of these adducts with respect to piperacillin-protein binding and the relationship with time, concentration and the antidrug antibody response.

(a)

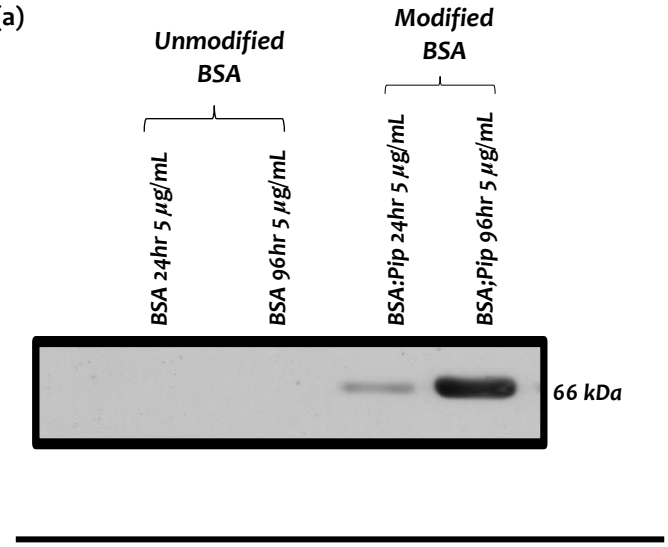
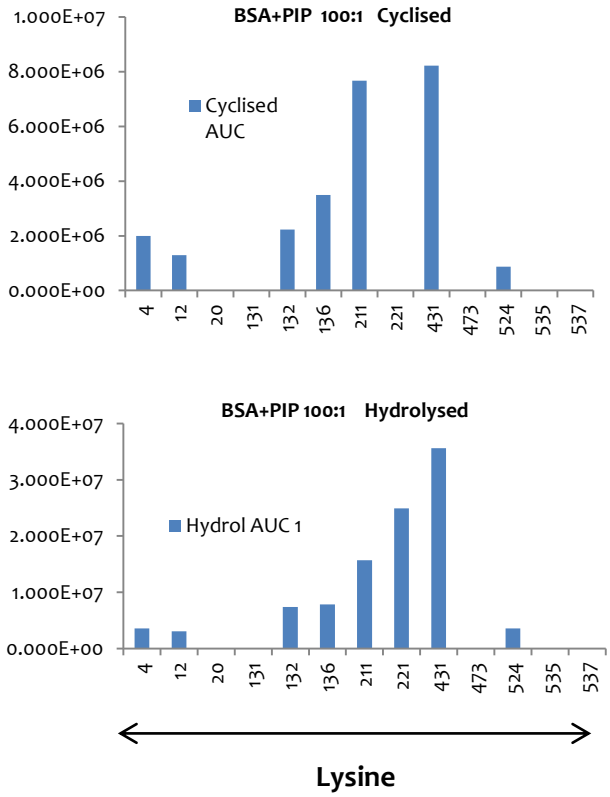


Figure 4.2.

Summary of piperacillin BSA binding discussed in detail in chapter 3.

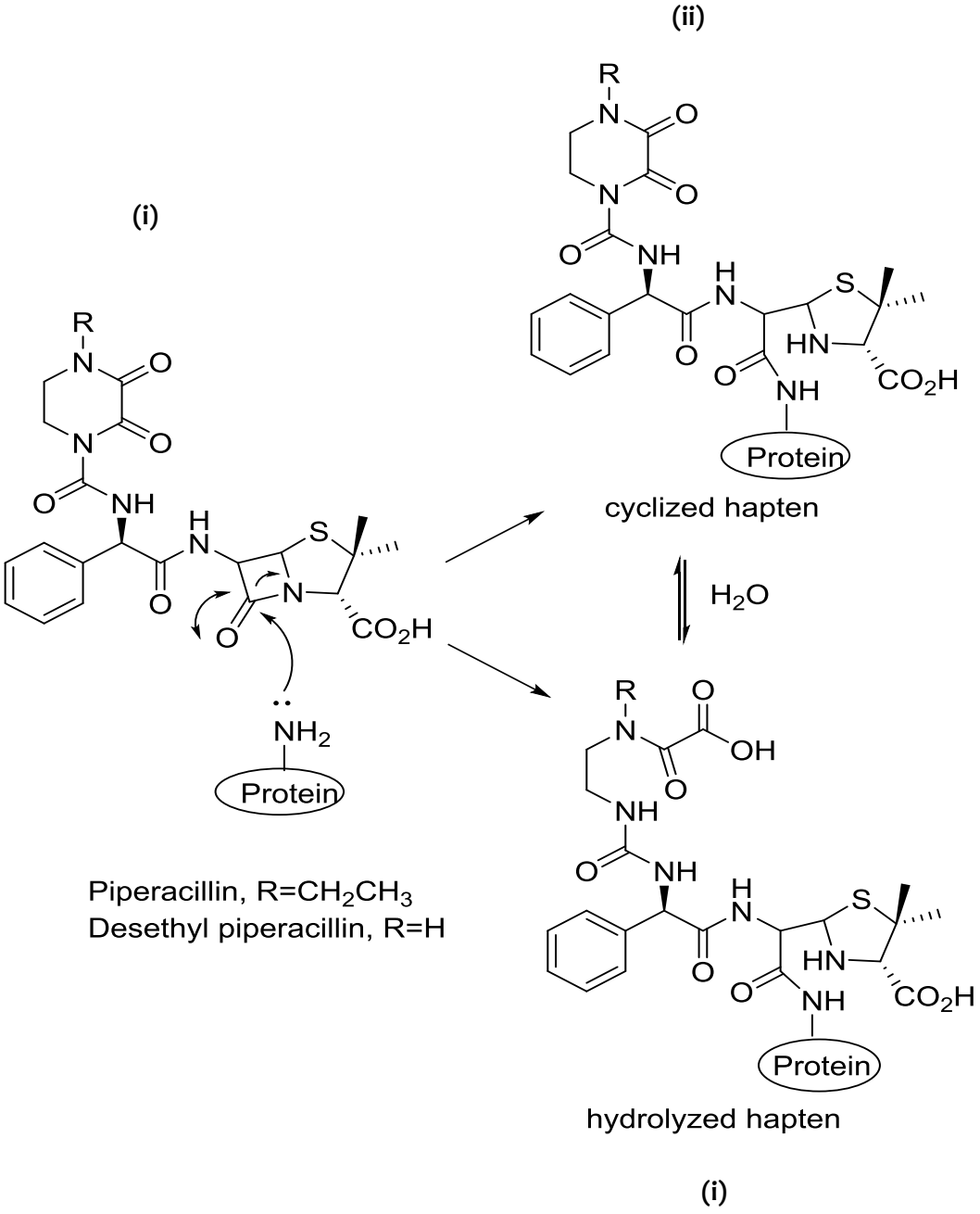
(a) SDS-PAGE characterization of the time-dependent formation of Piperacillin-BSA adduct. SDS-PAGE western blot was used to characterize the time dependent Piperacillin-BSA binding. BSA and piperacillin were combined at a molar ratio of 1:50 then incubated for 24 hrs and 96 hrs respectively at 37°C. The enhanced binding exhibited at the 96 h time point led to its characterization and use in the hapten inhibition assays using plasma and culture supernatants as discussed in chapter 3. This same conjugates were employed in this chapter.

(b)



(b) Mass spectrometric characterization of cyclised and hydrolysed forms of piperacillin haptens bound covalently to BSA.

Graphs showing the extent of lysine residue modifications by cyclised and hydrolysed piperacillin, at Piperacillin-BSA molar ratio of 100:1. Analysis was



(c) Chemical structures showing points of modification and insertion of functional groups (R) of (ii) cyclised and (iii) hydrolysed piperacillin-BSA adducts formed from the (iii) primary piperacillin structure ($R=CH_2CH_3$) and desethyl piperacillin (R=H) adducts.

4.2.2 Epitope profiling of piperacillin-BSA conjugate using mass spectrometry

Piperacillin-BSA conjugates with varying hapten densities were synthesized as previously described in the materials and methods section (see chapter 2). SDS-PAGE analysis of the conjugates revealed bands at 66 kDa using 1:1, 1:5, 1:10, 1:20, 1:50 and 1:100 - of BSA to piperacillin. SDS-PAGE analysis exhibited increased intensity in the bands. The conjugation of piperacillin to BSA occurred in a dose-dependent fashion (Figure 4.3)

A screen of the amino acid targets modified by piperacillin adduction were assessed using mass spectrometry analysis. The semi-quantitative analysis of individual lysine residue modification was then carried out. The comparison of individual lysine residue modifications across the different molar ratios was possible but comparison of separate modified lysine residues was not possible due to their differences in ionisation. Epitope profiles showing the individual lysine residues modified by piperacillin and the relative level of binding at each site derived from the mass spectrometric analysis of 96 hr BSA:Pip conjugates are shown in figure 4.4. The conjugates were prepared at various molar ratios ranging from 1:1 to 1:100. Lysine modifications were observed on 8 (eight) lysine residues, specifically residues at positions 4, 12, 132, 136, 211, 221, 431, and 524. The magnitude of individual modifications observed were found to increase as the BSA-Piperacillin molar ratio increased i.e. from 1:1 to 100:1, with the peptide containing the Lys431 residue appearing to ionise better than other residues at all the molar ratios (Figure 4.4). Importantly an increase in the total number of sites modified was not observed as the drug:protein ratio increased.

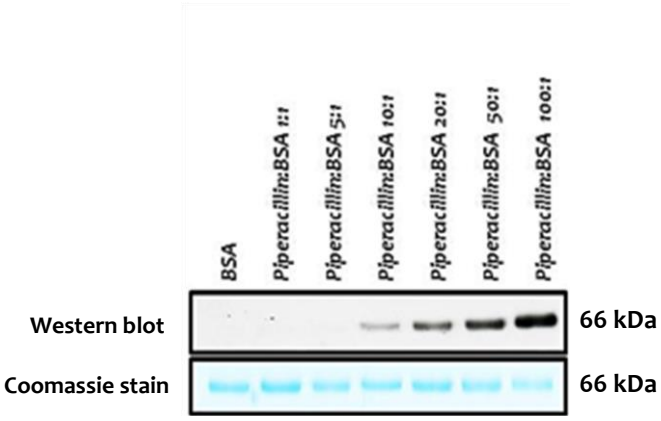


Figure 4.3. SDS-PAGE confirmation of Piperacillin-BSA binding. SDS-PAGE western blot was used to characterize the binding in various Piperacillin-BSA adducts at different molar ratios. Drug and BSA were combined at 1:1, 5:1, 10:1, 20:1, 50:1 and 100:1 Piperacillin-BSA molar ratios then incubated for 96 hours at 37°C. Adducts were then precipitated with methanol, western blotting performed and subsequently incubated overnight at 4°C in the presence of anti-penicillin mouse monoclonal antibody then goat anti-mouse secondary antibody prior to development and exposure in the dark room to confirm drug-protein adduction.

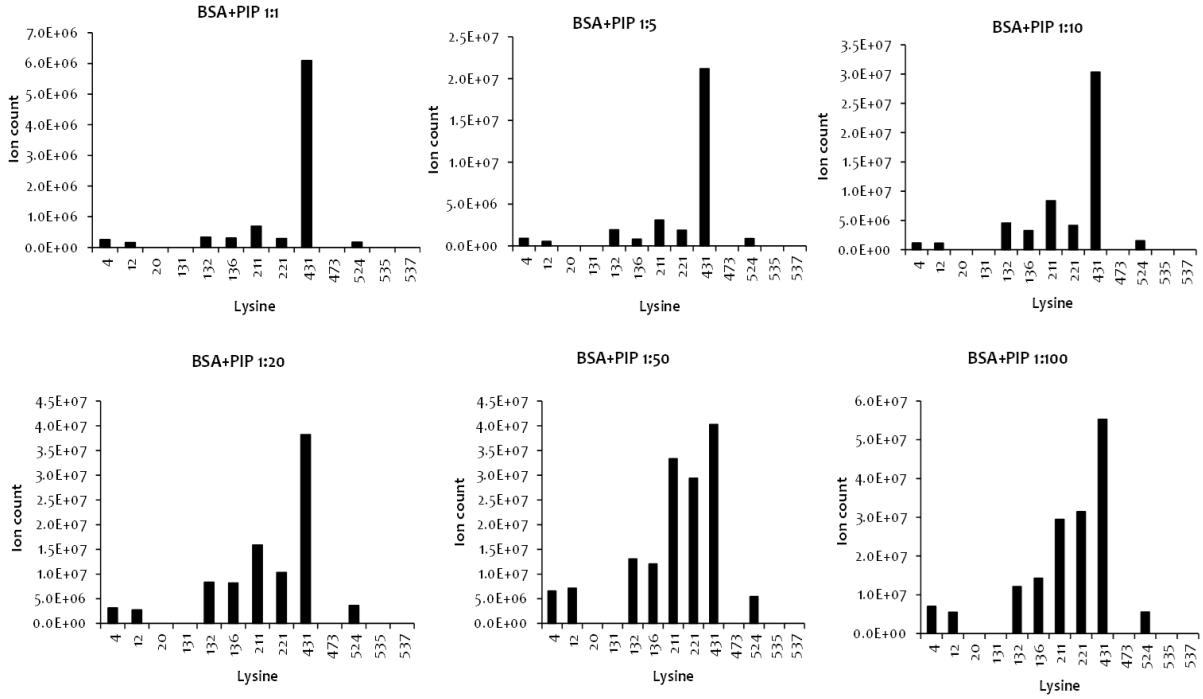


Figure 4.4 Epitope profiles at different BSA-Piperacillin hapten densities. The semi-quantitative comparison of individual lysine residues across different molar ratios was performed. BSA-Piperacillin adducts formed at molar ratios of 1:1 to 1:100 were digested with trypsin, denatured and desalted. The epitope profiles show a concentration dependent increase in individual lysine residue modification from 1:1 to 1:100.

4.2.3 *In vitro* concentration dependency of piperacillin-BSA binding

The overall modification of BSA with piperacillin (1:1 to 1:100) *in vitro* shows a concentration dependency with the highest ratio of drug to protein showing the highest level of modification (Figure 4.5 a). This is consistent with the concentration dependent increase in adduct formation which is observed at each individual lysine group (figure 4.5b).

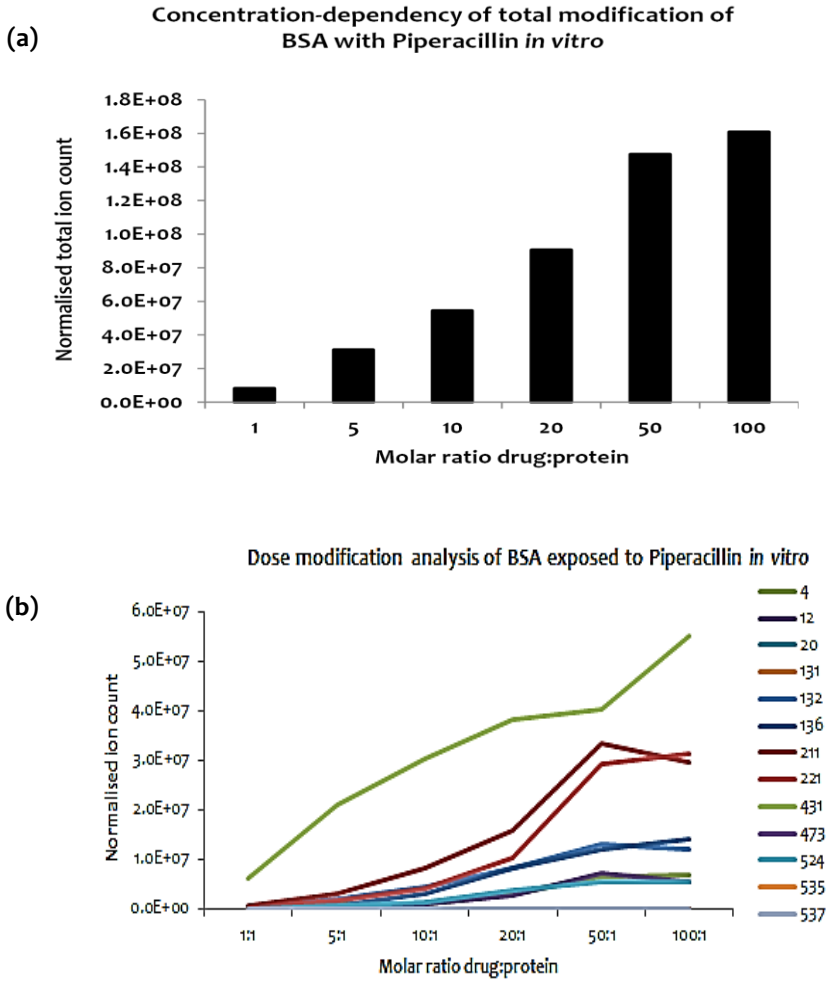


Figure 4.5 *In vitro* analysis of the modification of lysine residues in Piperacillin-BSA conjugate. (a) Piperacillin concentration-dependent modification of BSA (b) Total level of piperacillin-BSA modification at each ratio of drug:protein for individual lysine residues. Values were calculated by adding levels of binding detected at the 8 sites of drug modification.

4.2.4 Average molar quantification of Piperacillin-BSA conjugates

MALDI-TOF analysis showed the corresponding masses of the various conjugates at their different molar ratios. The masses observed at the different molar ratios were 66528.98 for the unmodified protein (Piperacillin-BSA 0:1), then 66740.73, 67029.0, 67499.0, 67499.33 and 67430.38 for the modified conjugates, 1:1, 10:1, 20:1, 50:1, and 100:1 respectively (Figure 4.6).

The mass values obtained from the MALDI-TOF analysis were then used to determine the density of the hapten bound to the carrier protein molecule. Determination was carried out via comparison of the variations in molecular weight of the different conjugates (1:1 to 100:1). The result obtained shows the hapten densities of the conjugates increasing with an increase in the piperacillin-BSA molar ratio, from 0.41 hapten molecules at 1:1 to 4 molecules at a ratio of 100:1 (Table 1). These values are considered to represent an estimate of the average molar quantification of drug:protein binding in piperacillin-BSA conjugates.

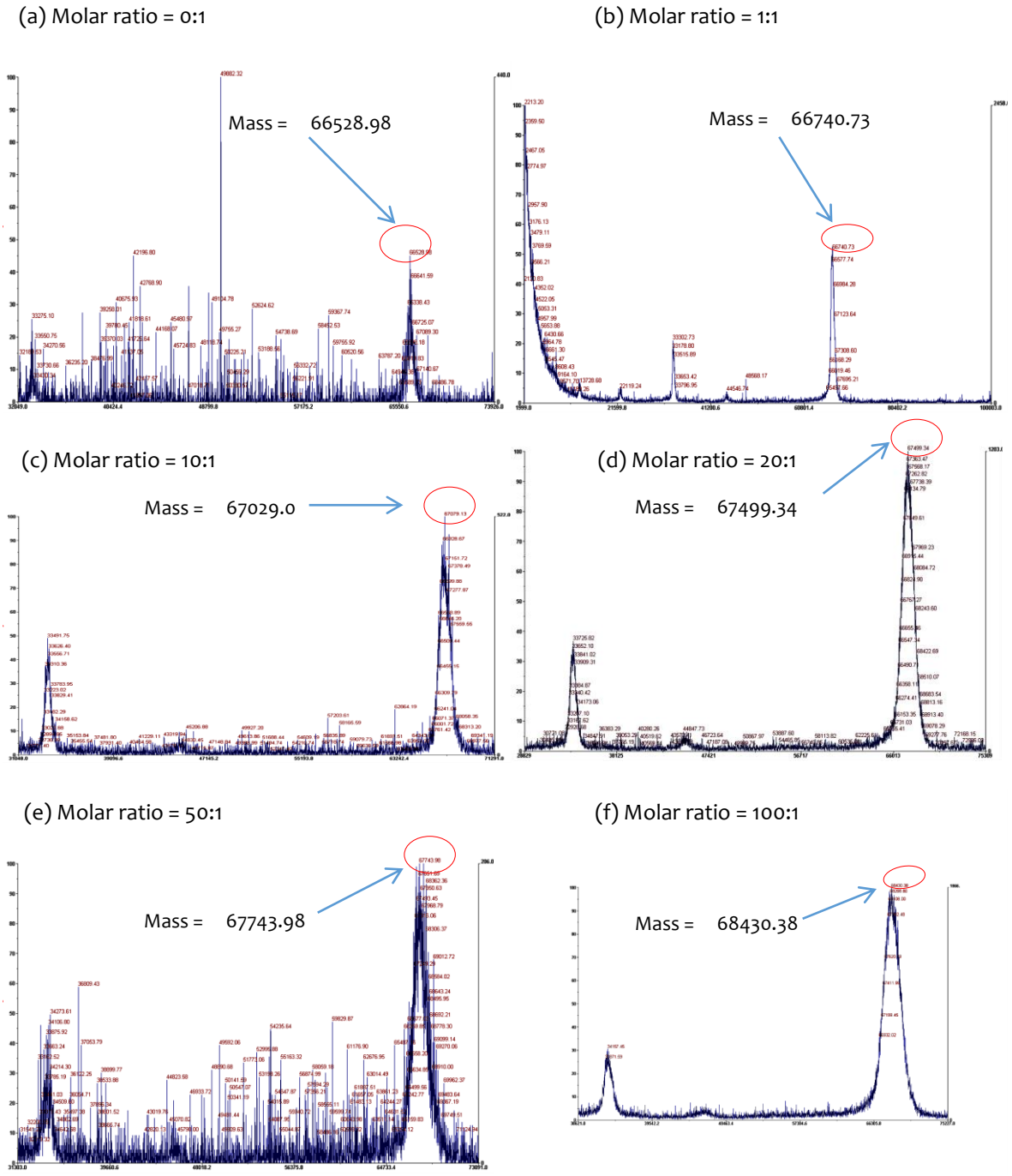


Figure 4.6. Spectral images of the molar quantification of piperacillin-BSA conjugates using matrix assisted laser desorption ionisation (MALDI). Undigested Piperacillin-BSA conjugates (0.8 µl) at molar ratios (1:1 to 1:100) was placed on a MALDI plate spot. (CHCA) matrix (0.8 µl) was then placed onto the sample, ensuring proper mixture of the matrix and sample before use. Calibration was achieved by the addition of 0.8 µl of the calibration control which also contained matrix. This was placed in between the sample spots. The MALDI plate was then allowed to dry before being loaded into the MALDI. The desired number of hits and traces were accumulated using Voyager control panel software.

Table 4.1 Average molar quantification of piperacillin-BSA conjugates. The observed molecular mass values from the MALDI - TOF was obtained, and these values were used to determine the mass variations detected from 0:1 to 100:1. The ratio of the variations to the molecular mass of piperacillin (ΔM/Mh) produced the hapten density, which was found to increase as the Piperacillin-BSA ratio increased. This value is roughly indicative of the number of piperacillin molecules that bind each BSA molecule after conjugation.

Molar ratio Piperacillin-BSA	Observed molecular mass (Da)	Mass variation (ΔM)	ΔM/Mh (hapten density)
0:1	66,528.98	0	0 (0)
1:1	66,740.73	211.75	0.41 (0)
10:1	67,029.0	500.20	0.97 (1)
20:1	67,499.34	970.36	1.88 (2)
50:1	67,743.98	1,215.0	2.35 (2)
100:1	68,430.38	1,901.40	3.67 (4)

4.2.5 Assessment of the molar ratio-dependent binding of piperacillin-specific antibody to protein conjugates

The hapten inhibition assay was employed (see chapter 2) to assess the response of antidrug antibodies to different densities of piperacillin haptens bound covalently to BSA.

Using plasma samples from piperacillin hypersensitive patients and patients from the cohort that express high levels of piperacillin-specific IgG, we employed these piperacillin conjugates of varying hapten densities (BSA-piperacillin- ratios of 1:1 to 1:100). The difference observed in IgG secretion between the piperacillin treated and hapten inhibited groups at each molar ratio was taken as the amount of piperacillin specific IgG. Results were obtained following a single treatment per molar ratio; hence standard deviations are not presented and formal statistical analyses were not performed on individual patients.The results suggested a direct correlation between the hapten density (degree of conjugation) and the subsequent antibody response. Immunogenic responses varied depending on the individual sample used. Responses were observable from 1:5<1:10<1:20<1:50<1:100 molar ratios in (Figure 4.7 a, and 4.7 c) 1:1<1:5<1:10<1:20<1:50<1:100 in (Figure 4.7 b) and 1:10<1:20<1:50<1:10 (Figure 4.7 c). The anti-drug antibody dependency on total hapten density also shows a molar ratio dependent increase in antibody-drug binding (Figure 4.7 e) which is consistent with the individual increases observed in figures 4.7 (a), (b), (c) and (d).

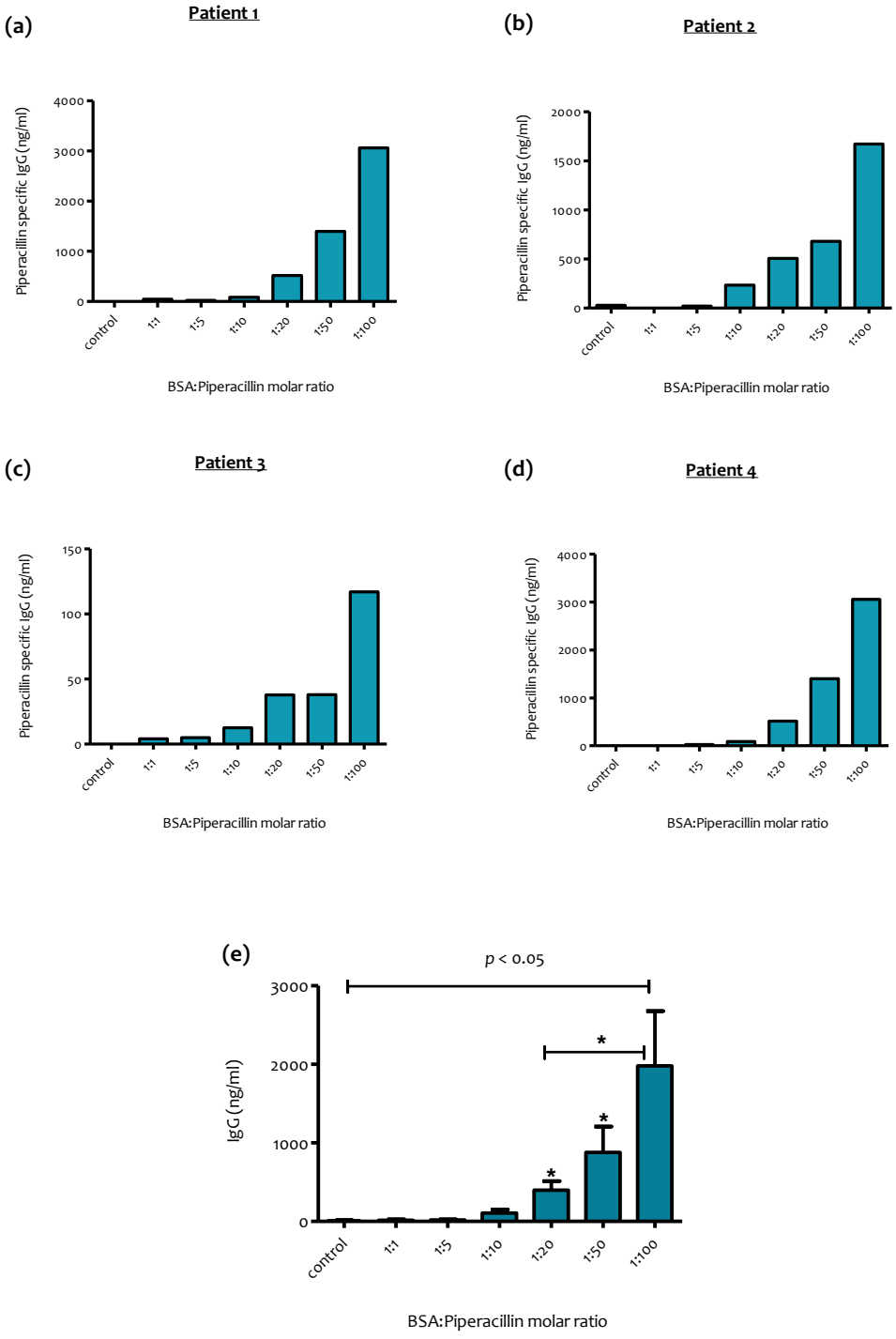


Figure 4.7 Molar ratio-dependent binding of antibody to β-lactam conjugates. Using plasma from 2 patients from the cohort expressing high levels of piperacillin-specific IgG (a), (b) and 2 piperacillin hypersensitive patients (c and d) Piperacillin-BSA conjugates of varying hapten densities (Piperacillin-BSA ratios of 1:1 to 1:100) were employed to assess the *in vitro* molar ratio-dependent antibody binding (e) Combined results of the three hypersensitive patients. Data were analysed by the Students T test to compare the difference between means with p≤0.05 considered as significant. * denotes p = 0.01 to 0.04.

4.2.6 Epitope profile of amoxicillin (AMOX), benzyl penicillin (BP), flucloxacillin (FLU), penicillin V (PEN V) and aztreonam (AZT) BSA adducts

BSA:drug (piperacillin, amoxicillin, benzyl penicillin, flucloxacillin, penicillin V and aztreonam) conjugates with varying hapten densities were synthesized as described previously in materials and methods (see chapter 2). SDS-PAGE analysis of the conjugates showed drug-protein binding - via the detection of visible bands at 66 kDa for all drugs tested. Epitope profiles derived from the mass spectrometric analysis of these conjugates show different levels of lysine (Lys) residue modification. Furthermore, drug-dependent modification profiles of Lys residues were observed. Amoxicillin, benzyl penicillin and flucloxacillin were all found to modify Lys residues at positions 4, 12, 131, 211, 431, 523, and 524, but in addition also lysine residues at positions 20, 473 and 537 were modified for amoxicillin and BP. Position 132 was common to Flu and BP and Lys535 was only modified with flucloxacillin. Aztreonam and penicillin V showed the least number of modified lysine residues with Lys136, 211 and 524 bound by aztreonam and Lys 211, 431, and 524 bound by penicillin V. The Lys residue at position 211 was the only modification that was consistent in all adducts. Due to the low abundance of the FLU-BSA conjugate, the true extent of modification possible may have not been defined. (Figure 4.8 (a) to 4.8(e))

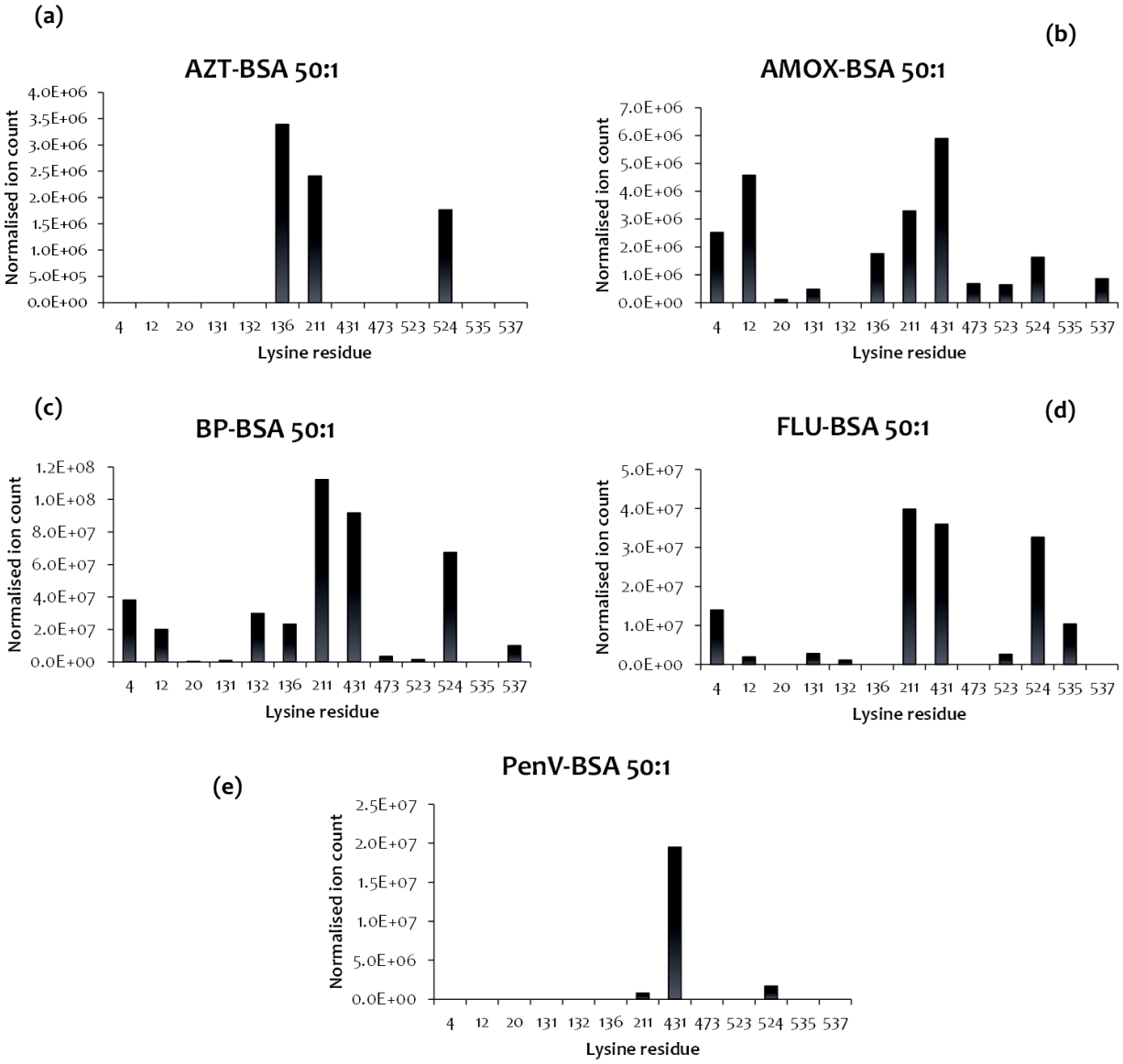


Figure 4.8. Epitope profiles for β-lactam-BSA adducts. β-lactam-BSA adducts with molar ratios of 50:1 were digested with trypsin, denatured and desalted then characterized by mass spectrometry.

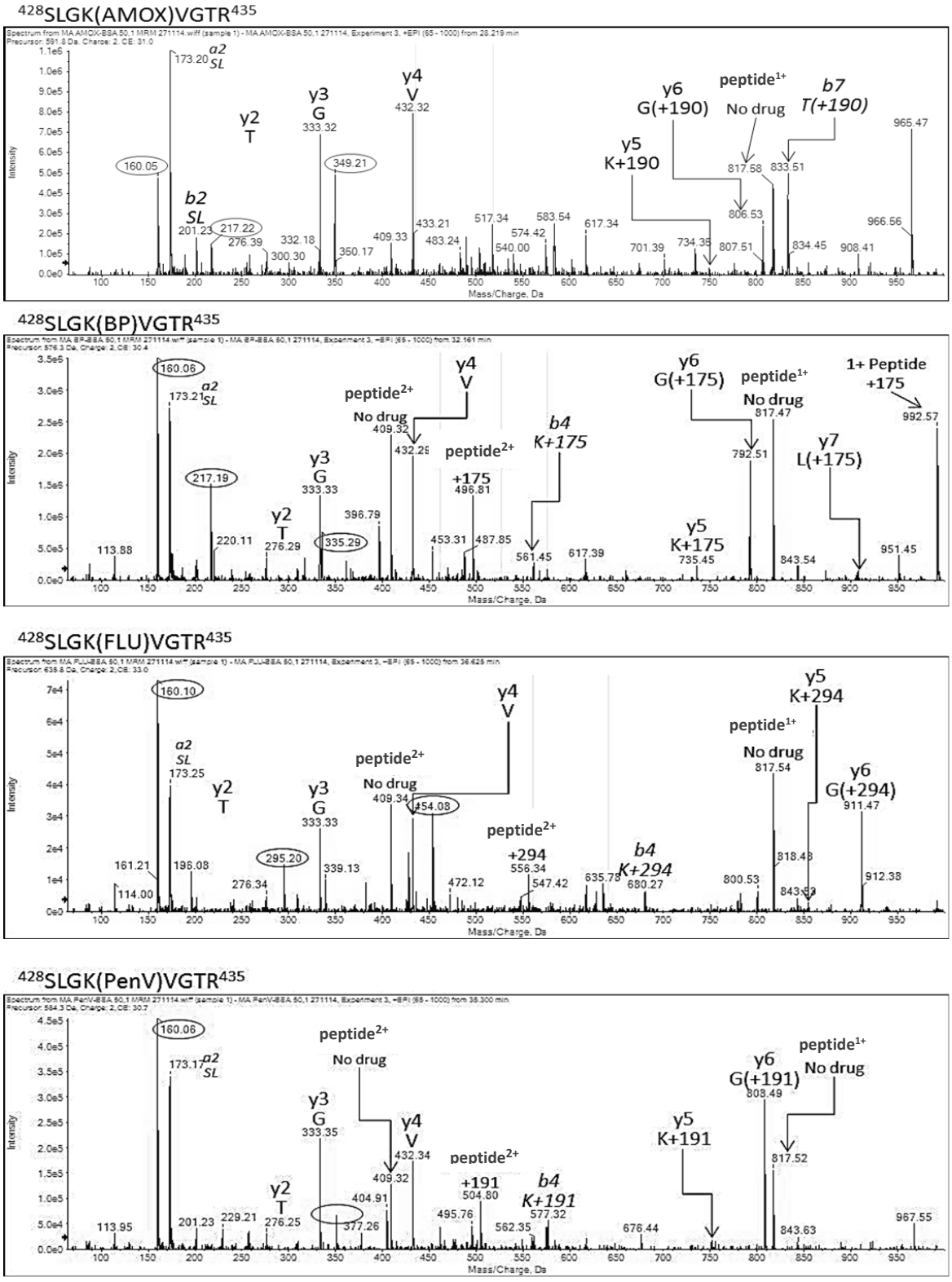
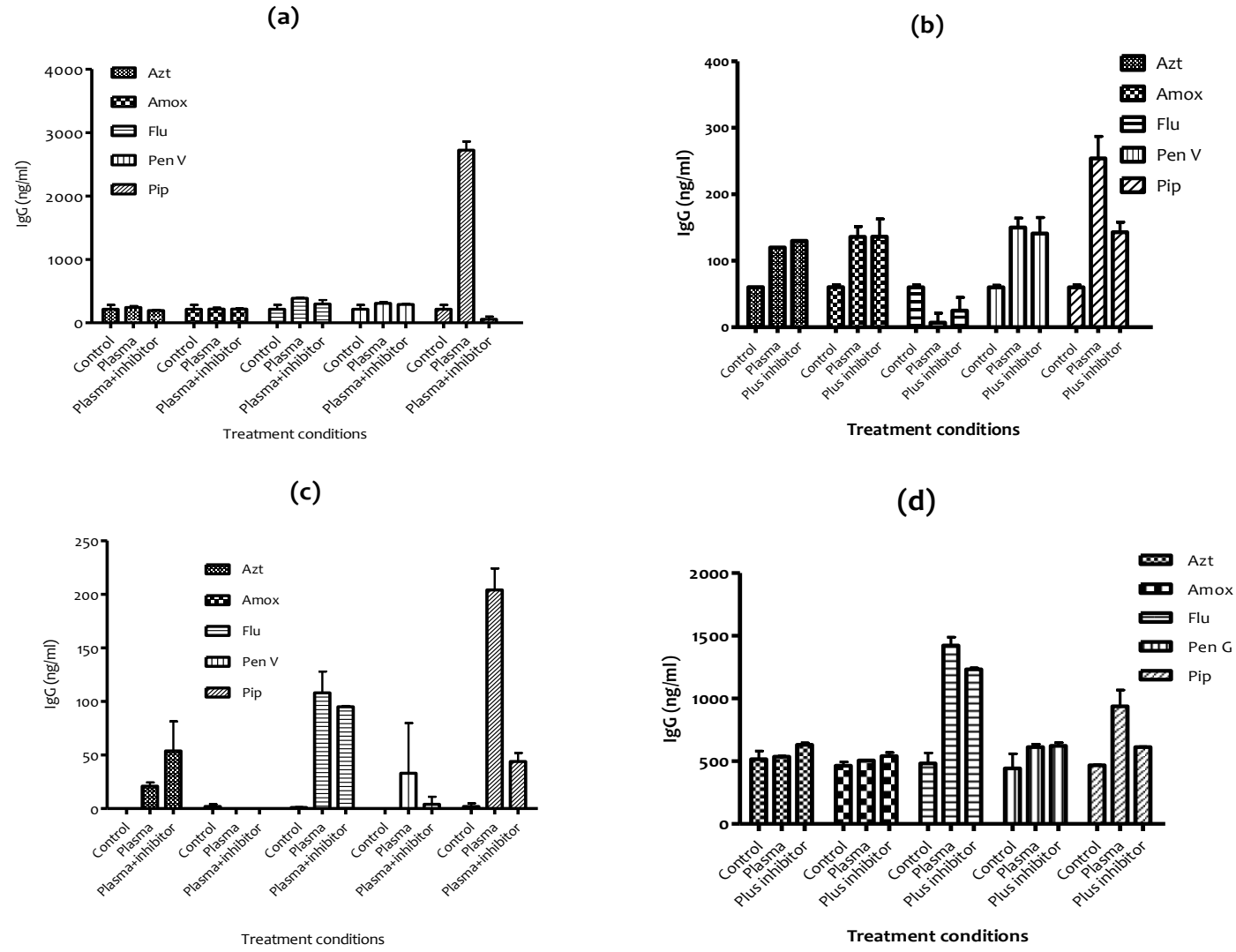


Figure 4.9 MS/MS spectral images of modified BSA:amoxicillin, BSA:benzylpenicillin, BSA:flucloxacillin and BSA:penicillin V, adducts. Modification of LYS431 with different penicillins consistent with the presence of a characteristic fragment ions at *m/z* 160 and drug-specific fragment ions.

4.2.7 Determination of β-lactam cross-reactivity of antipiperacillin antibodies

The hapten inhibition assay as described in materials and methods (chapter 2) was employed to determine the cross reactivity of anti-drug antibodies in hypersensitive patient plasma samples. Conjugates were prepared using five different β-lactam antibiotics; amoxicillin (Amox), aztreonam (Azt), flucloxacillin (Flu), penicillin G (Pen V), and piperacillin (Pip). These conjugates were assessed for antidrug antibody detection using samples obtained from 4 (four) hypersensitive patients, earlier confirmed to have substantial amounts of circulating anti-piperacillin IgG antibodies (figure 4.10 a). The results obtained indicate substantial antibody binding to piperacillin-BSA conjugates in all patients and subsequent inhibition of the response following the addition of an excess of soluble piperacillin to block hapten binding. The antibody binding noted with piperacillin-BSA was notably absent when amoxicillin, aztreonam, flucloxacillin and penicillin V - BSA conjugates were employed. A clear indication of the specificity of the antidrug antibodies and absence of any form of cross reactivity between these drug haptens and piperacillin. The cleavage of the β-lactam ring and the presence of a thiazolidine ring are common to all adducts formed using the various β-lactam antibiotics (See schematic in figure (3.7a-b) and 4.2c). Therefore, the ability of circulating antidrug antibodies to bind to piperacillin conjugates alone and also be inhibited by free piperacillin suggested that in contrast to other β-lactams, antibody recognition of the piperacillin adduct was via the piperacillin side-chain.

The detection of low levels of IgG binding to the flucloxacillin adduct likely represents non-specific binding as it was not blocked with the addition of soluble drug.



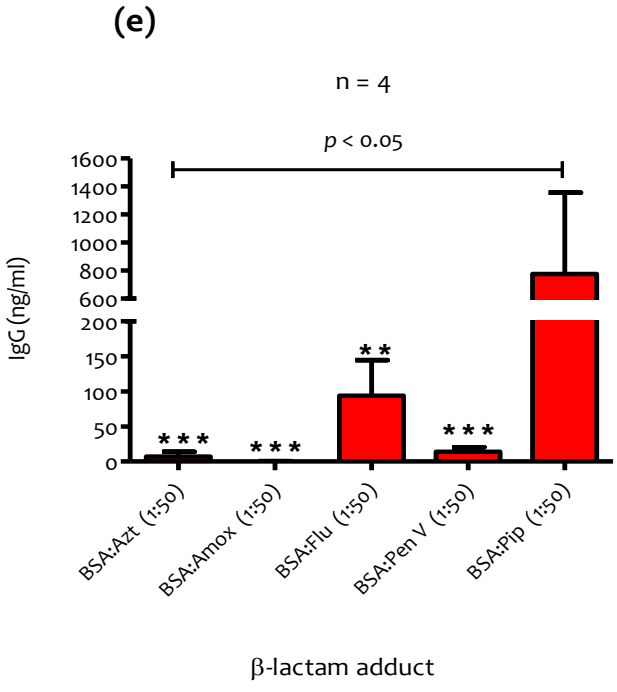


Figure 4.10.

Hapten inhibition ELISA employing unmodified and β-lactam modified BSA to assess cross-reactivity to other β-lactams.

The presence of antidrug antibodies in plasma of four patients (a, b, c, and d) was measured by ELISA. Flat bottom high binding 96 well plates were pre-coated overnight with the BSA-drug adducts for aztreonam (Azt), amoxicillin (Amox), flucloxacillin (Flu), penicillin G (Pen G) and piperacillin (Pip) as an antigen. Plasma (100 µl) was added, with 1 mM soluble drug employed as the inhibitor. Colour was developed by reaction of HRP and TMB substrate. The reaction was stopped with 0.18M H_2SO_4. Data was analysed by the Students T test (e) to compare the difference between means with $p \leq 0.05$ considered as significant. ** denotes $p < 0.01$; *** denotes $p < 0.001$).

4.3 Discussion

Piperacillin-protein adducts with different levels of hapten binding were generated initially to enhance antigen binding in the ELISA. However, these adducts also permitted the characterization of the relationship between the extent of amino acid residue modification and the strength of antibody binding. HSA was initially used to prepare the piperacillin adducts but the high backgrounds led to the selection and use of BSA as the protein backbone. This choice was further justified by the similar masses and structure of adducts as determined by mass spec analysis, the detection of the same lysine targets that are modified with piperacillin in HSA (Whitaker, Meng et al. 2011, Jenkins, Yaseen et al. 2013) and the high backgrounds associated with the other alternative protein analyzed (see chapter 3).

The ratio at which drugs and proteins are conjugated has previously been shown to influence the nature of the antibodies induced by the hapten, with an increase in the ratio usually bringing about an increase in the strength and specificity of the immune responses (Marco, 1995). Western blot analysis using an established protocol (Whitaker, Meng et al. 2011) was employed to confirm adduct formation. The mass differences between conjugates compared to the molecular weight of BSA at 66 kDa, was too small to provide significant measureable migration distances using electrophoretic analysis. Nevertheless, the results show that the number of piperacillin molecules bound to BSA increased in a linear fashion with an increase in the ratio between piperacillin and BSA. Molar ratios ranging from 1:10 to 1:100 showed an increase in adduction based on data following quantification with MALDI-TOF with a corresponding increase in the ratio showing hapten densities of 1, 2, 2 and 4 (approximate values from table 4.1). BSA-modified ciprofloxacillin at a molar ratio of 1:800 has previously

shown binding of up to 30 ciprofloxacin molecules. Only 26 out of the 56 lysine residues exist on the BSA molecule surface, suggesting that ciprofloxacin is able to bind other amino acid residues apart from lysine (Rosenoer VM 1977, Hirayama, Akashi et al. 1990). An important point here is that protein targets of reactive electrophilic metabolites usually contain strong nucleophilic sites. Subsequently, in addition to lysine amines, these protein targets include cysteine thiol, histidine imidazoles, and protein *N*-terminal amines. The accessibility of these nucleophilic groups, protein localization within the cell, the nature of the reactive metabolite and post adduction chemistry may alter the nature of the final adduct (Labenski, Fisher et al. 2009). This further affirms the possibility of adduction to specific lysine residues and other multiple nucleophilic sites by ciprofloxacin and most likely other drugs and chemicals.

Herein, with piperacillin such high drug-protein adducts were not used by virtue of previous studies where plasma kinetics were studied (Paton, Mander et al. 1982, Gath, Charles et al. 1995, Roder, Frimodt-Moller et al. 1995) and molar ratio of drug to HSA in plasma was determined to be 1:16, with adducts also detectable at a 1:10 molar ratio. Clinically however, patients are also administered multiple doses of the drug which leads to accumulation of the drug-protein conjugate despite the half-life of HSA (Anderson and Anderson 2002). Thus, drug to BSA molar ratios from 1:1-1:100 used in our study were thought to be reasonable relative to those obtainable *in vivo*.

From our results we can see that an increase in the protein-hapten molar ratio led to an increase in the hapten density of conjugates, which has a great influence on the binding affinity of piperacillin-specific antibodies, whereby an optimum molar ratio of piperacillin-BSA conjugates was able to induce significant piperacillin binding.

Western blot analysis showed that the incubation time of drug with protein had a direct effect on the level of adduct formation. A significant increase in piperacillin hapten binding was observed at the 96 hours compared to the 24 h incubation time (4.2.2).

Following the concentration - and time-dependent characterization of the conjugates, protein modifications which arose due to adduction were assessed by LC-MS/MS. The mass spectrophotometric analysis of piperacillin-HSA conjugates formed *in vitro* has been previously carried out (Whitaker, Meng et al. 2011) and was further confirmed in our study with the predicted mass of 517 amu identified (MS/MS profile of piperacillin binding using BSA conjugates). Similarly, modification was seen at the 8 lysine residues in BSA at positions 4, 12, 131, 132, 136, 211, 431, 524, and 537. The relative level of modification at each lysine residue increased significantly with an increase in drug:protein molar ratio. Collectively, these data coincide with that seen previously with HSA binding, and though Lys 4 and Lys 12 appear to be the only similar residues that were modified in both HSA and BSA adducts, in actual fact 4 additional piperacillin modified residues namely Lys 136, Lys 211, Lys 431, and Lys 524 were identified which ended up with different residue numbers due to their different primary amino acid sequences in BSA and HSA.

The hapten mechanism of drug hypersensitivity discussed in chapter 1 illustrates the significance of drugs/hapten-protein adduction in precipitating hypersensitivity reactions. Previously, the relationship between epitope density has been related to adduct disposition and immunogenicity using albumin-dinitrofluorobenzene (DNFB) conjugates. Low levels of modification were not detected by IgG antibodies, while the high levels of modification lead to protein degradation. Hence a bell-shaped curve was observed when epitope density was plotted against IgG binding (Park, Tingle et al. 1987). Subsequently our study has shown that in addition to the hapten density, the increase in the extent of

lysine residue modification, with an increase in the molar ratio rather than only the numbers of modified lysine residues are factors that define the extent of antibody binding. How these factors affect immunogenicity though, still remains unresolved.

Clinical cross-reactivity has been found to occur in hypersensitive patients with different classes of drugs including the β-lactam antibiotics. The cross-reactivity in drug hypersensitivity reactions between amoxicillin and cephadroxil, and to sulfasalazine and sulfamethoxazole both sulfonamides are clinically well documented examples (Sastre, Quijano et al. 1996, Zawodniak, Lochmatter et al. 2010). It is possible that the drug haptens bind to the same protein targets. This promiscuity could be due to the structural similarities which these molecules possess (Oldstone 1998), though unrelated ligands have also been implicated (James, Roversi et al. 2003) with data obtained from previous studies pointing to binding involving specific hydrogen bonds dependent upon the chemistry of the cross reactant and the availability of complementary antibody residues (James and Tawfik 2003).

Previous studies employing piperacillin-specific T-cell clones have shown the selective proliferative capacity with piperacillin and not other β-lactams which share the penicilloyl core structure or drugs that share similar side chains (El-Ghaiesh, Monshi et al. 2012). To show the specificity of the anti-drug antibodies for piperacillin, adducts were generated using five different β-lactams. Mass spectrometric analysis and indirect competitive ELISA analysis were carried out to define antibody binding and relate the individual lysine residue modifications to the immune response. The total absence of responses with Azt, Amox, BP and Pen V or the significant reduction as seen with flucloxacillin of anti-piperacillin IgG binding to drug–protein conjugates was indicative of the absence of cross

reactivity with piperacillin. Thus, piperacillin-specific antibodies are highly drug-specific and must recognize both the penicilloyl structure and specific side chain of piperacillin; thus, restricting interactions with similar drugs that bear different side chains.

Epitope profiles showed similar lysine residues were modified in amoxicillin, benzylpenicillin and flucloxacillin conjugates though the extent of ionisation of the various modified peptides varied. The modifications identified were specific for each hapten and the subsequent effect on peptide ionisation; hence the various intensities could not be compared across the different drugs. Also the reactivity of flucloxacillin with its propensity to cause protein damage at high concentration leading to losses during sample processing could be regarded as a factor that may have led to the low abundance of the FLU-BSA conjugate.

In this chapter drug conjugates have been used to characterize piperacillin protein binding and assess the variable effects of time and concentration on the drug-specific antibody binding. Collectively, the data begins to describe the cellular processes that determine the specificity of piperacillin antibody binding and enhances the understanding of the humoral response in piperacillin hypersensitive patients.

CHAPTER 5

CHARACTERISATION OF PIPERACILLIN-SPECIFIC T-CELL RESPONSES AND IgG ANTIBODIES IN PATIENTS WITH CF.

Table of Contents

Figures

Tables

5.0 Introduction

In earlier chapters of this thesis I tried to enumerate the molecular mechanisms that underlie the development of drug hypersensitivity. I have also reviewed the obvious relationship which exists between the presence of an existing pathology, which in this case is cystic fibrosis (CF), and the increased expression of a form of these reactions due to β-lactam administration, specifically piperacillin. Literature to support these facts has been documented in chapters 1 and 2 of this thesis.

In prospective cohort studies, the design, subject recruitment and data collection are usually the primary responsibility of the investigator(s). Data is also usually collected before any treatment or outcomes of interest have been developed by the subjects. These kinds of studies have important applications in epidemiology and in clinical studies (Bhandari and A. 2009, Bookwala, Hussain et al. 2011). After collection of the initial data these subjects are usually followed up longitudinally to the conclusion of the study.

Previously, prospective studies have been conducted utilising cohorts of patients with CF to study certain disease parameters such as disease prevalence, patient mortality and survival, disease genetic correlation, and also effects on pregnancy and surgical interventions. A collection of these studies have been enumerated in the table below (Table 5.1). None of these 12 studies was tailored towards understanding the immunological basis of the adverse reactions observed in patients exposed to β-lactam antibiotics, a scenario we have tried to address in our study.

Table 5.1. Prospective studies utilising CF patient cohorts to study incidence, prevalence, survival and mortality, and genetic studies.

Year	Reference	Nature of study	Number of patients in the study
1996	(Kosorok, Wei et al.)	Incidence of CF	19,781
1998	(Hamosh, FitzSimmons et al.)		17,755
2002	(Scotet, Gillet et al.)		543
2007	(Bellis, Cazes et al.)		4,104
1982	(Kollberg)	Prevalence, survival and mortality studies	116
2003	(O'Connor, Quinton et al.)		23,817
2007	(Bellis, Cazes et al.)		4,104
2008	(Castellani, Cuppens et al.)		8,284
1998	(Feingold, Guilloud-Bataille et al.)	Genetic studies	15,620
2001	(Koch, Cuppens et al.)		8,963
2004	(Badet, Bellis et al.)		3,220
2006	(McKone, Goss et al.)		16,651

The above studies employed large cohorts, but the nature of the data generated is limited. Apart from the few genetic-based studies very little molecular information can be gleaned from the studies in relation to drug-related reactions and other clinical parameters. This form of analysis would require the assessment of select molecular characteristics of the CF cohort, enabling us to characterise the contributions or not of various cellular subsets and their secretions to the onset of the adverse events.

An improved understanding of drug hypersensitivity requires a multidisciplinary approach that relates immune responses to clinical outcome using samples obtained prior to a reaction, in the acute phase and longitudinally as the patient recovers. This form of investigation can only be conducted through establishment of a prospective clinical study in combination with specialized laboratory facilities to explore immune function. Piperacillin hypersensitivity in patients with CF represents one of the few reactions where sufficient samples can be collected prospectively from patients with a clinically relevant adverse drug reaction. The data presented in this chapter represents the first analysis of a four year study involving 80 patients. On recruitment an initial naïve or tolerant–blood sample was obtained; PBMC and plasma were isolated from whole blood and frozen at -150°C and -80°C respectively. Repeated blood samples were obtained on commencement of each piperacillin treatment course as well as on clinical diagnosis of a hypersensitivity reaction. Finally, a post-hypersensitivity sample was obtained after the resolution of clinical symptoms of hypersensitivity reactions. From each blood donation piperacillin-specific T-cell responses were assessed using the lymphocytes transformation test (LTT) and/or interferon-gamma (IFN-γ) ELISpot. Total and drug-specific IgG was measured by ELISA. The main objective of this chapter was to establish when piperacillin-specific T-cell and antibody responses are detectable in drug-exposed patients and to establish their possible relevance in the onset of drug hypersensitivity.

5.1 Methods

Refer to chapter 2 sections 2.4.

5.2 Results

5.2.1 Sample collection and presentation of data

Eighty patients undergoing therapy with intravenous piperacillin were recruited for this study. Blood samples were collected at different time points of piperacillin administration; before the administration of piperacillin (pre), during piperacillin drug therapy (mid), at the end of therapy (end) or clinical diagnosis of a hypersensitivity reaction (reaction). Finally, a retrospective sample (retro) was obtained at least 1 month after allergy diagnosis. LTT results were positive for 32 patients (one or more samples) during the course of piperacillin administration; while the remaining 48 patient samples were negative. Forty-three patients had complete course samples taken. From this cohort the number of piperacillin courses per patient ranged from a single course to a maximum of 5 courses. Twelve patients produced positive LTTs within the duration of the first course, 3 patients by the second course and 3 by the third course (figure 5.1). Positive LTTs were observed from as early as the first day after administration to 14 days and also at the resumption of treatment up to 14 months after the first course of piperacillin was given. Positive LTTs were also observed up to 8 weeks post desensitisation.

Since the clinical study is still ongoing it is not possible to access clinical details of patients. However a hypersensitivity reaction was diagnosed clinically following the discovery of one or more of the following, delayed onset maculopapular/urticarial rash, fever and arthralgia. Firstly we used the LTT to relate T-cell responsiveness to the allergy/tolerant phenotype. Patients that produced negative LTTs throughout were deemed negative hence tolerant. Patients that initially had positive LTTs from the pre

sample but subsequently had a negative end or reaction sample were classified as negative. Patients that produced positive LTTs throughout were deemed positive hence hypersensitive. Patients that initially had negative LTTs from the pre sample but subsequently had a positive end or reaction sample were classified as positive (irrespective of a clinical diagnosis of allergy).

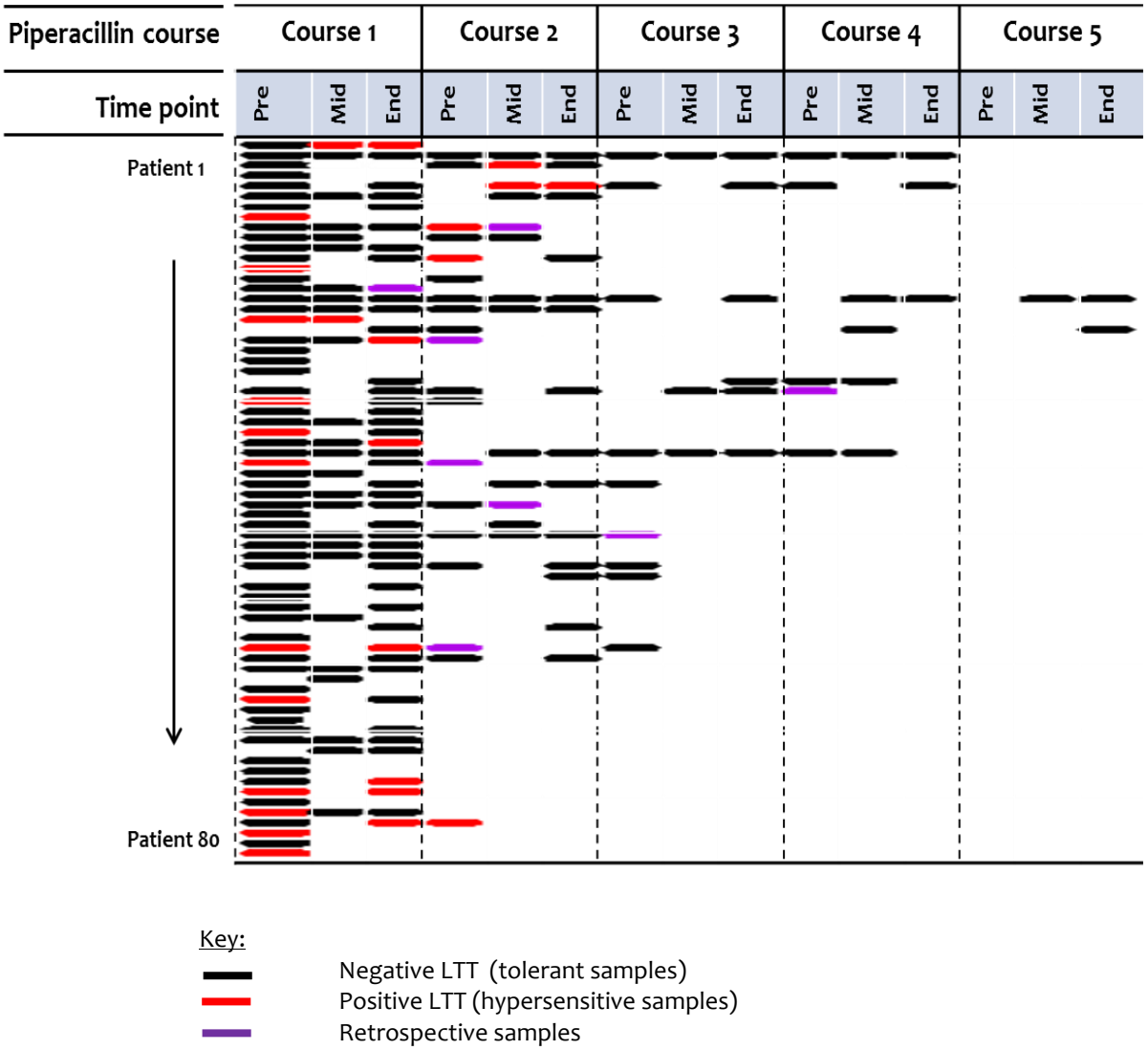


Figure 5.1 Summary of total number of piperacillin courses administered to each patient. Blood samples were obtained during each course of piperacillin administration from CF patients at different time points. The figure shows the total number of courses administered to each patient, the number of courses leading to the detection of hypersensitivity and the point at which tolerance (black bars) and hypersensitivity to piperacillin was detected in-course (red bars). Retrospective samples (purple bars) are indicated for some of the patients.

A total of 20 patients were clinically classified as allergic. From this group of patients 9 produced positive LTT results from which 5 were specifically from the allergy/reaction

sample, while 11 patient samples produced negative LTTs (figure 5.2). Importantly of the 11 negative LTTs with acute reaction samples, 65 patients are yet to donate a retrospective sample; hence, the specificity of the LTT cannot yet be assigned.

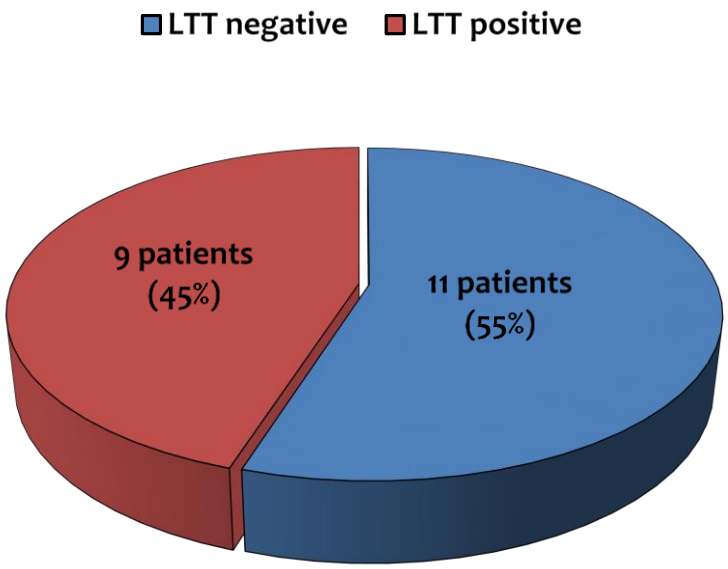


Figure 5.2 Relationship between clinical diagnosis of hypersensitivity and drug-specific T-cell responses. Pie chart showing the total number of patients clinically diagnosed with hypersensitivity and their drug specific responses.

The presence of clinically diagnosed hypersensitive patients without piperacillin-specific T-cell responses suggested that these patients may have been misdiagnosed or there could be a deficiency in the test used. Resolving these questions would require (i) the donation of the post reaction blood sample and (ii) the patients being rechallenged with a test dose of the drug. Individual courses were assessed to determine whether and how the detection of piperacillin-specific T–cells related to clinical outcome. The LTT results obtained from pre, mid, reaction and retrospective samples helped to define the stages during a course of piperacillin administration at which (i) tolerance was observed (ii) the

point when hypersensitivity was diagnosed and (iii) if these responses persisted retrospectively (figure 5.3 and 5.4).

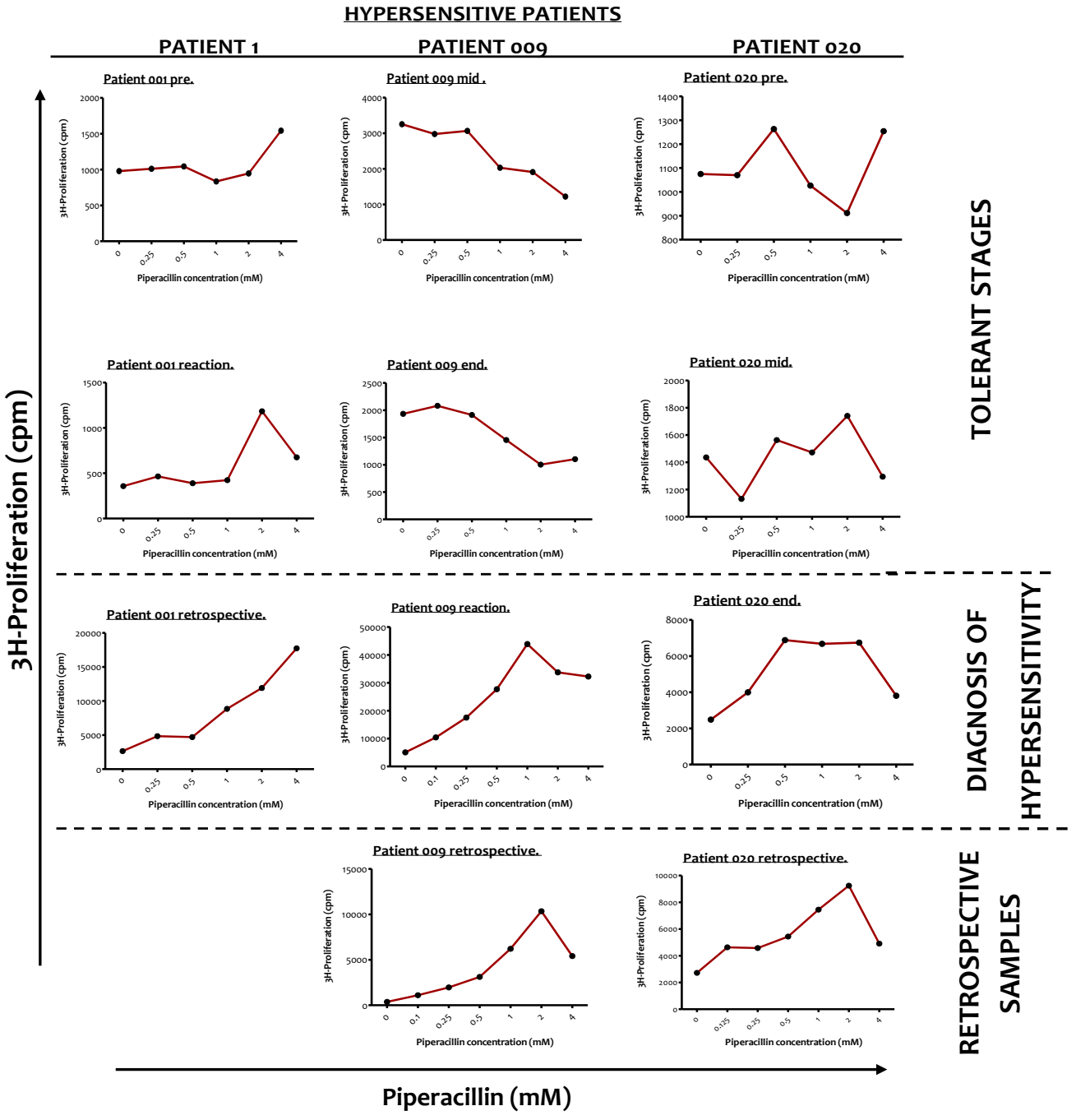


Figure 5.3 Assessment of piperacillin-specific T-cell responses from 3 hypersensitive patient's individual courses. LTT positive blood samples collected at pre, mid, end and retro time points were individually assessed to determine the relationship to the clinical outcome. Graphs show LTTs from three hypersensitive patients indicating the point at which tolerance ended and a hypersensitivity diagnosis was made via the LTT, and also if this status persisted retrospectively.

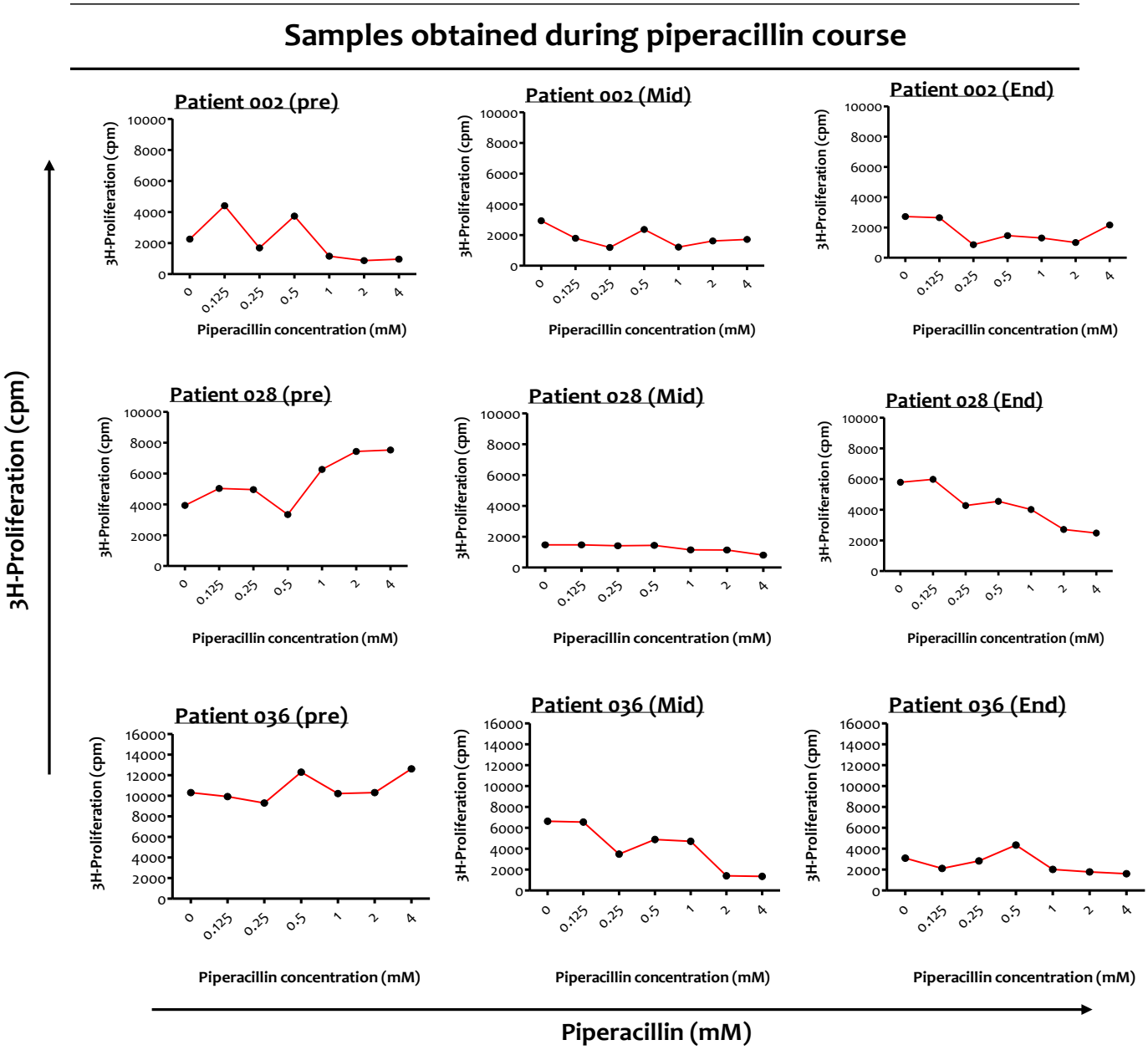


Figure 5.4 Assessment of individual courses from tolerant patients. LTT negative blood samples collected at pre, mid, end and retro time points were individually assessed to determine the relationship to the clinical outcome. Graphs show LTTs from three tolerant patients indicating the absence of a hypersensitivity diagnosis and the retrospective status.

A host of clinically diagnosed piperacillin tolerant patients presented consistent LTT profiles over multiple courses that reflected their non-responsive nature (figure 5.5). However a small number from this group of patients also developed positive piperacillin-specific T cell responses.

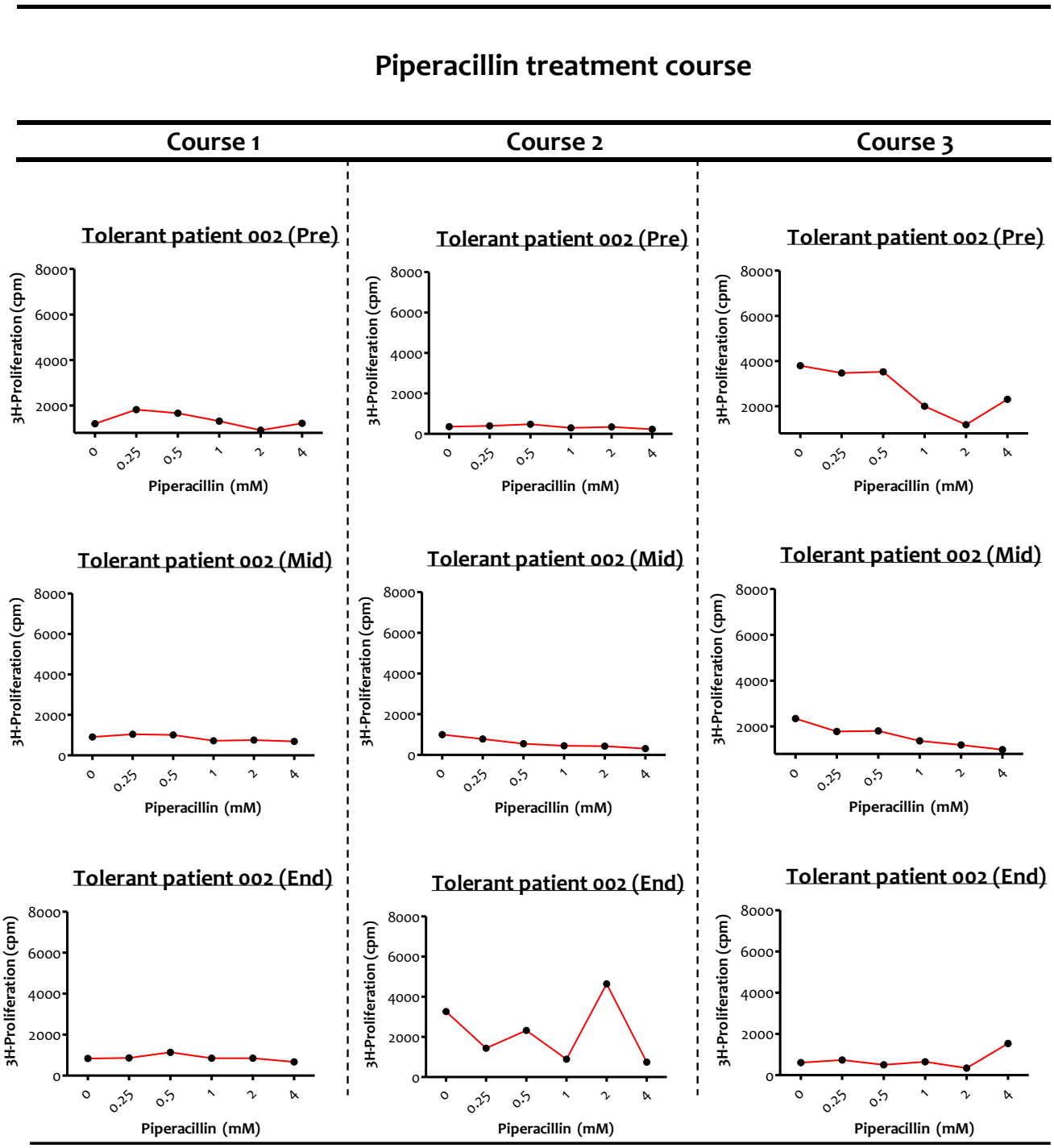


Figure 5.5 Representative LTT profile from clinically diagnosed tolerant patients. Figure shows representative LTT graphs from a single tolerant patient of 3 piperacillin administration courses. Consistent LTT profiles were observed over multiple courses of piperacillin administration with SI < 2.

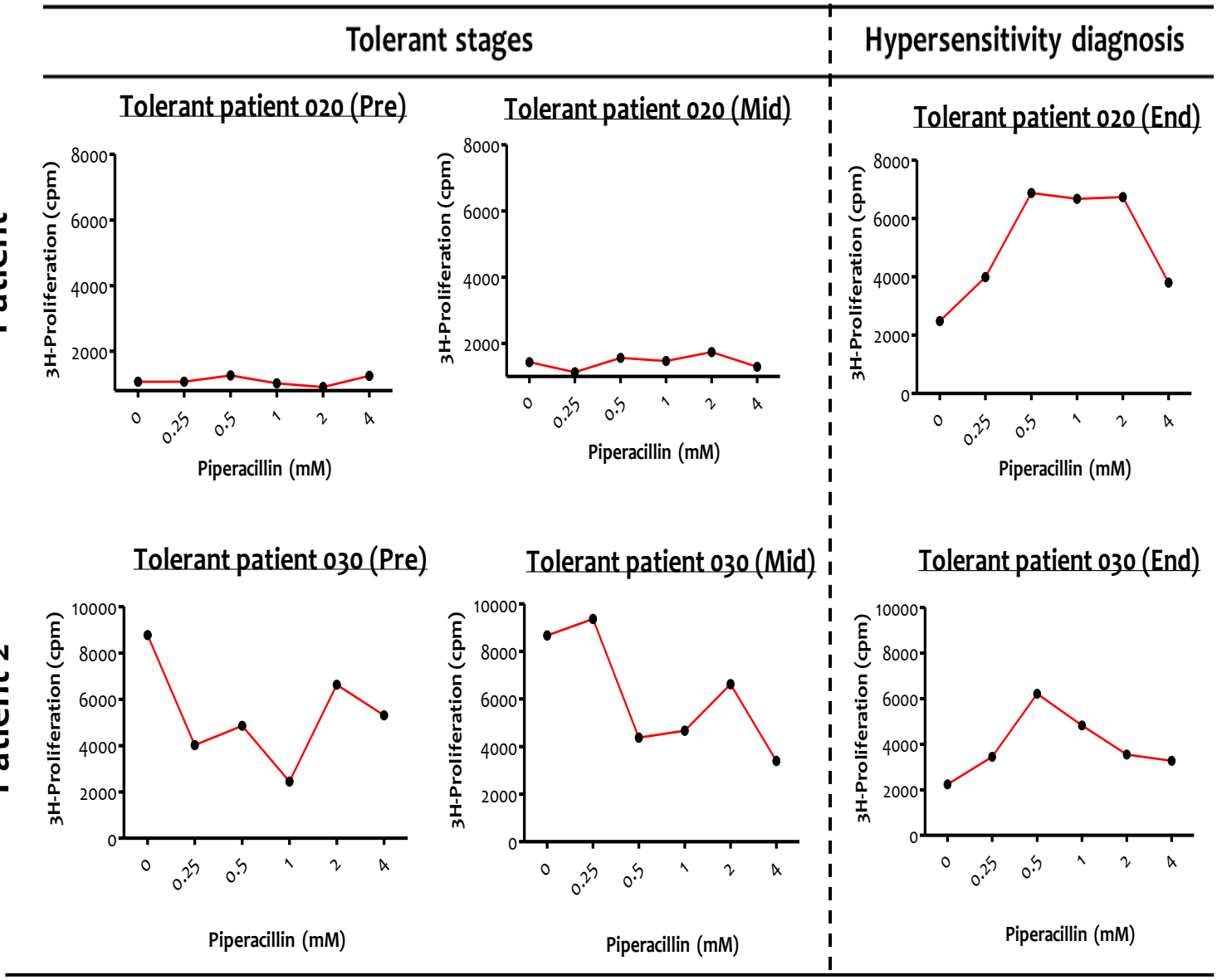


Figure 5.6 Clinically diagnosed patients with a positive LTT. Figure shows representative LTT profiles from clinically diagnosed tolerant patients. Over a single course of piperacillin administration, patients exhibited a tolerant profile at earlier time points but a hypersensitivity diagnosis at the terminal sample collection point (SI > 2).

5.2.2 Assessment of patients LTT status, with total and piperacillin-specific IgG secretion

Blood donations were divided according to the piperacillin-specific T-cell response and assayed for the expression of total and drug-specific IgG. There was no significant difference in total IgG between LTT positive and negative patients (Figure 5.7a) However, a significant difference (p <0.05) was observed in piperacillin-specific antibody between both groups with LTT positive samples expressing a higher average (Figure 5.7b). The kinetics of IgG detection was assessed in CF patients (Figure 5.8) and a cohort of patients that developed a clinically diagnosed reaction in the presence or absence of a drug-specific T-cell response (Figure 5.9). Two time points (Pre, and reaction samples) were assessed for their total IgG levels but no significant differences were found in the IgG levels between groups (Figure 5.9).

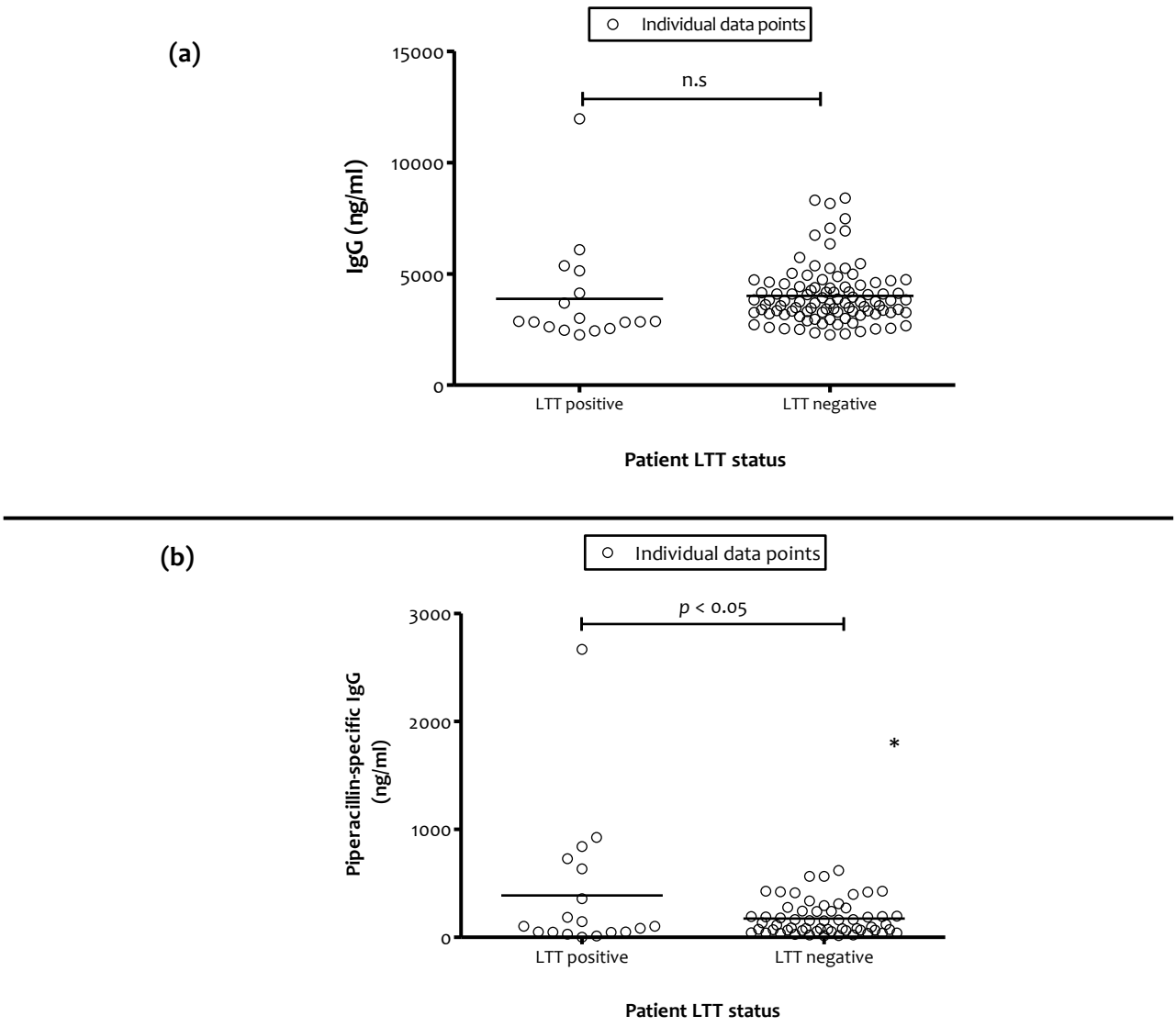


Figure 5.7 Drug-specific T-cell responses and the expression of drug-specific IgG. Patient blood samples were divided into LTT positive and negative samples Patient plasma was collected and total IgG and hapten inhibition ELISAs performed (a) Plasma was assessed for total IgG [LTT negative (n=92) and positive (n=23)] production and (b) piperacillin-specific antibody [LTT negative (n=74); LTT positive (n=21)]. Data were analysed by the Students T-test with $p < 0.05$ considered statistically significant (* denotes $p < 0.05$)

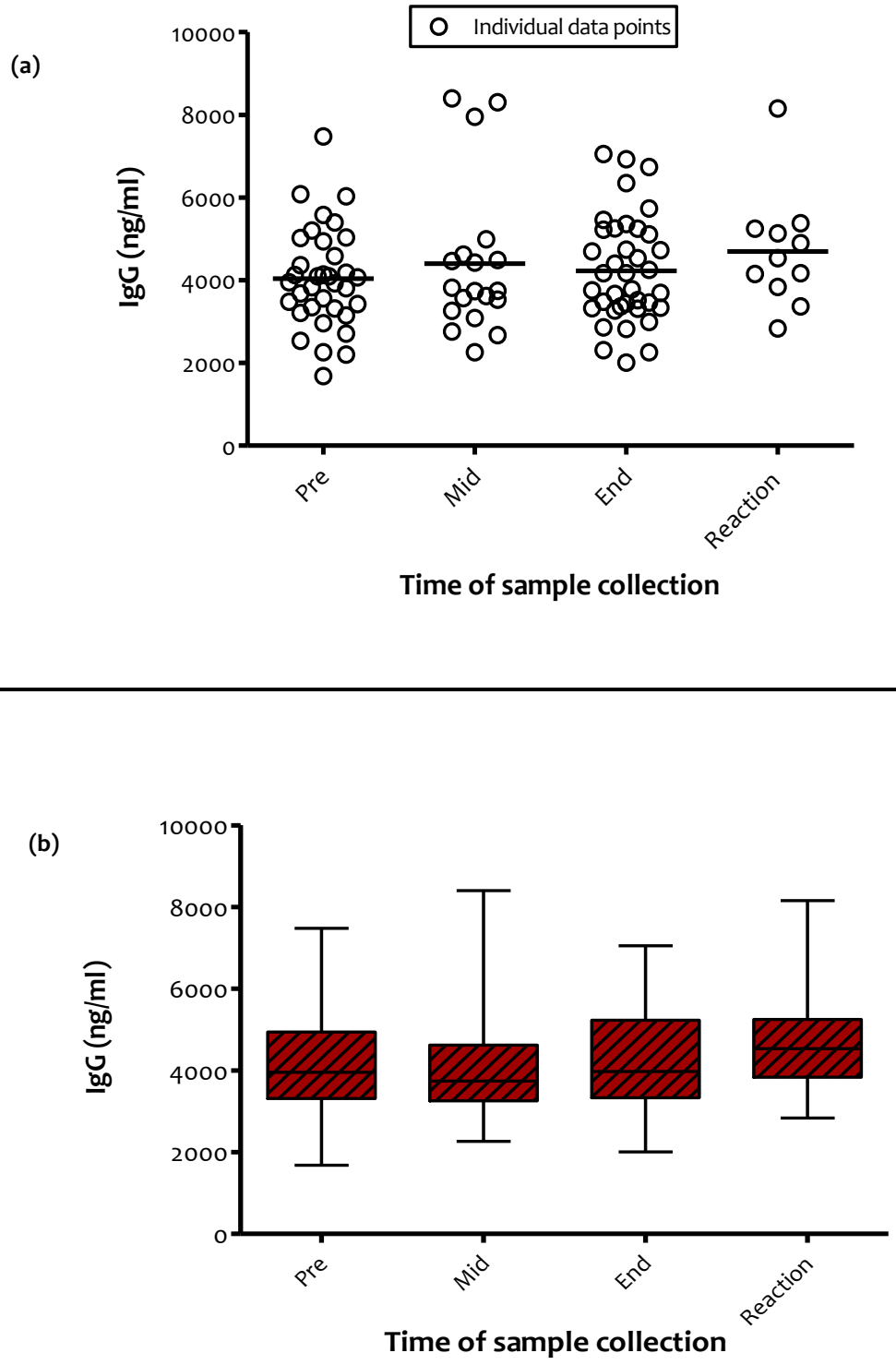


Figure 5.8 Kinetics of IgG detection in CF patients. Dot (a) and whisker plots (b) showing the total IgG secretion in CF patients being administered piperacillin. Plasma samples were obtained at four different time points namely from pre-drug treatment (pre) [n=36], a sample at halfway (mid) [n=19], through to the end of the course (end) [n=38] or at the clinical diagnosis of hypersensitivity (reaction)[n=11].

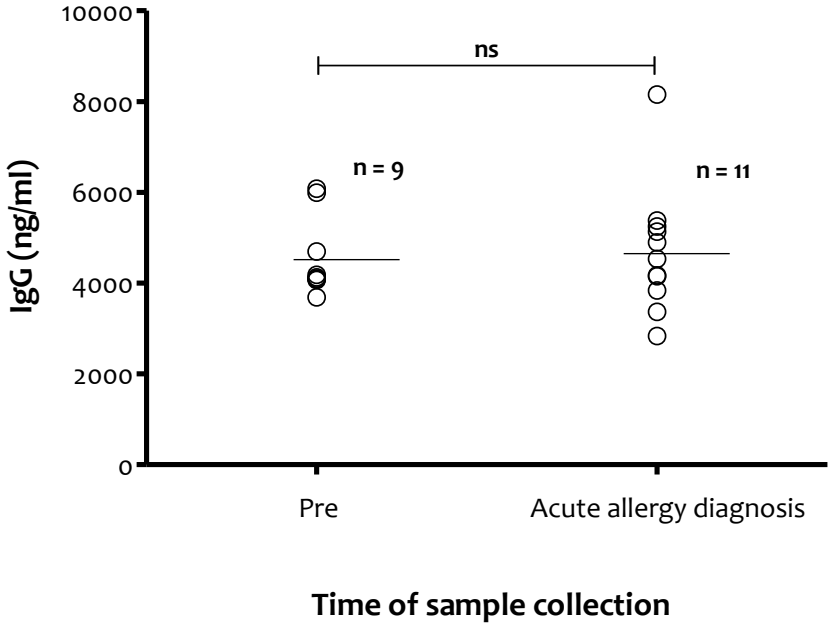


Figure 5.9 Kinetics of IgG detection in clinically diagnosed hypersensitive patients. Plasma samples from clinically diagnosed hypersensitive patients were assessed for IgG antibody using an ELISA. Individual data points were captured and the average concentration at each sample collection point determined. A students T-test was performed to determine the significance level ($p \leq 0.05$ was considered as statistically significant).

5.2.3 Characterisation of piperacillin-specific T-cell responses, total and piperacillin-specific IgG in patients undergoing desensitisation

T-cell responses and drug-specific IgG was measured in a cohort of patients undergoing desensitisation. Blood samples were collected from patient's pre-desensitisation (pre-des) and at different time points during desensitisation. LTTs were performed on these samples to determine T-cell responsiveness during desensitisation. However due to the inconsistent time points employed with desensitisation blood samples during collection (table 2) the 1h, and 24h post-desensitisation times were used for data analysis.

Table 5.2. Representative sample of LTT analysis of PBMC from desensitisation patients. Samples were collected from patients undergoing desensitisation at 6 different time points and assessed for Piperacillin-specific T-cell responses. Table shows some of the samples tested to present an overview of the time points used and the need to restrict analysis to only 1h and 24h.

	DESENSITISATION TIME POINT					
PATIENT ID	**Pre-Des**	**1hr**	**3hr**	**18hr**	**24hr**	**48hr**
019	—	—	NS	—	—	NS
024	—	**+**	NS	NS	**+**	NS
043	**+**	**+**	NS	NS	**+**	—
044	**+**	**+**	**+**	NS	—	NS
050	—	NS	NS	NS	NS	—

— = *Negative LTT result;* **+** *= Positive LTT result;* ***NS*** *= No sample received.*

Analysis of the total IgG level was performed on all patient desensitisation samples (figure 5.10), and with four specific patients that had a full complement of pre, 1h and 24h post desensitisation samples (figure 5.11). In both groups the total IgG was significantly higher pre-desensitisation than 24h post-desensitisation. This could possibly be attributed to two patients that possessed extremely high IgG levels which could be as a consequence of the drug, disease or interindividual variability.

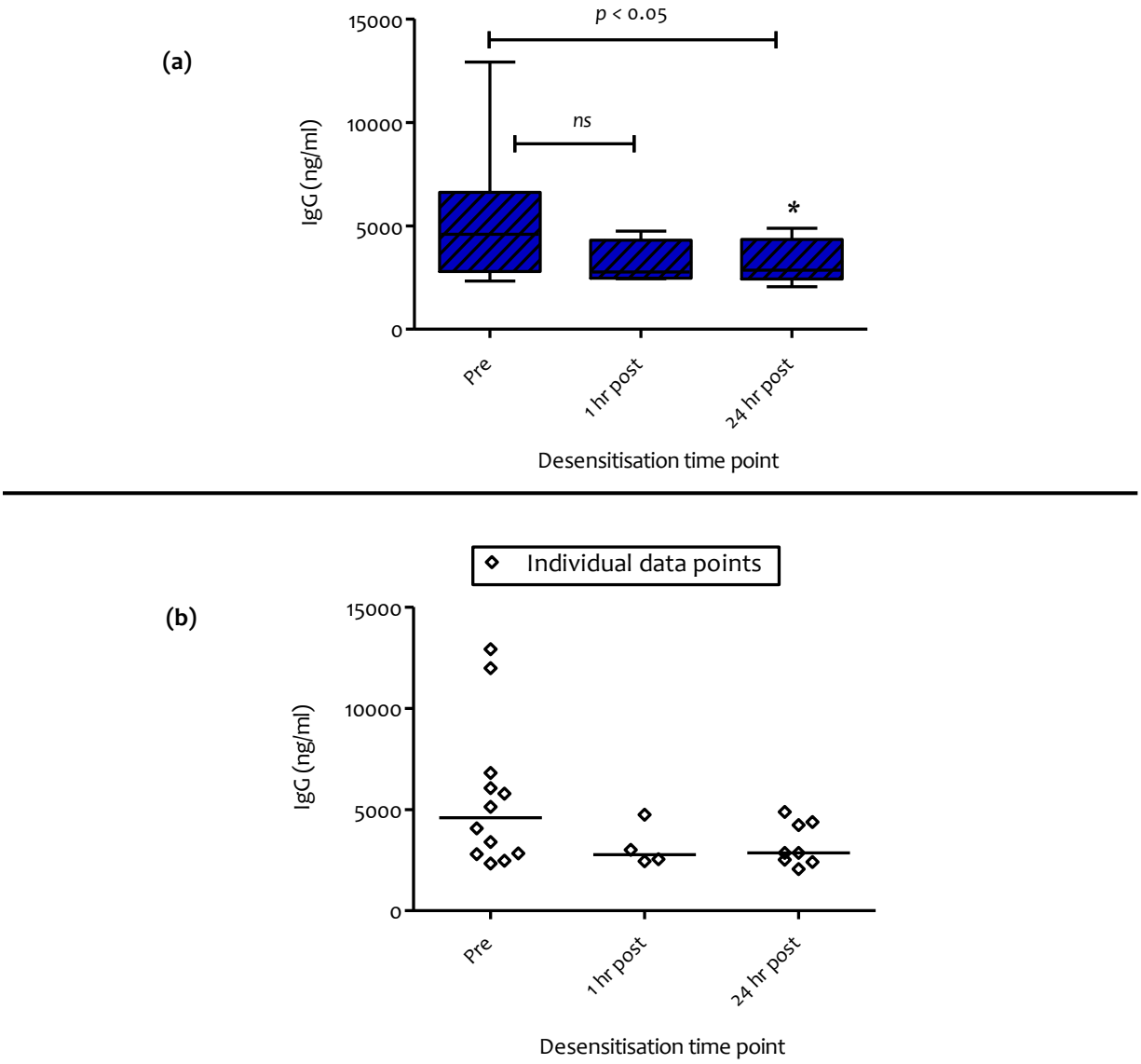


Figure 5.10 Analysis of total IgG level of all desensitisation samples. Total IgG ELISA whisker plots (a) and dot plots (b) showing total IgG levels in patients prior to desensitisation n = 12 , 1 hour (n = 4) and up to 24 hours post desensitisation to piperacillin (n = 8). Data were analysed by the Students T-test to compare the difference between means with p≤0.05 considered as significant (* denotes $p < 0.05$).

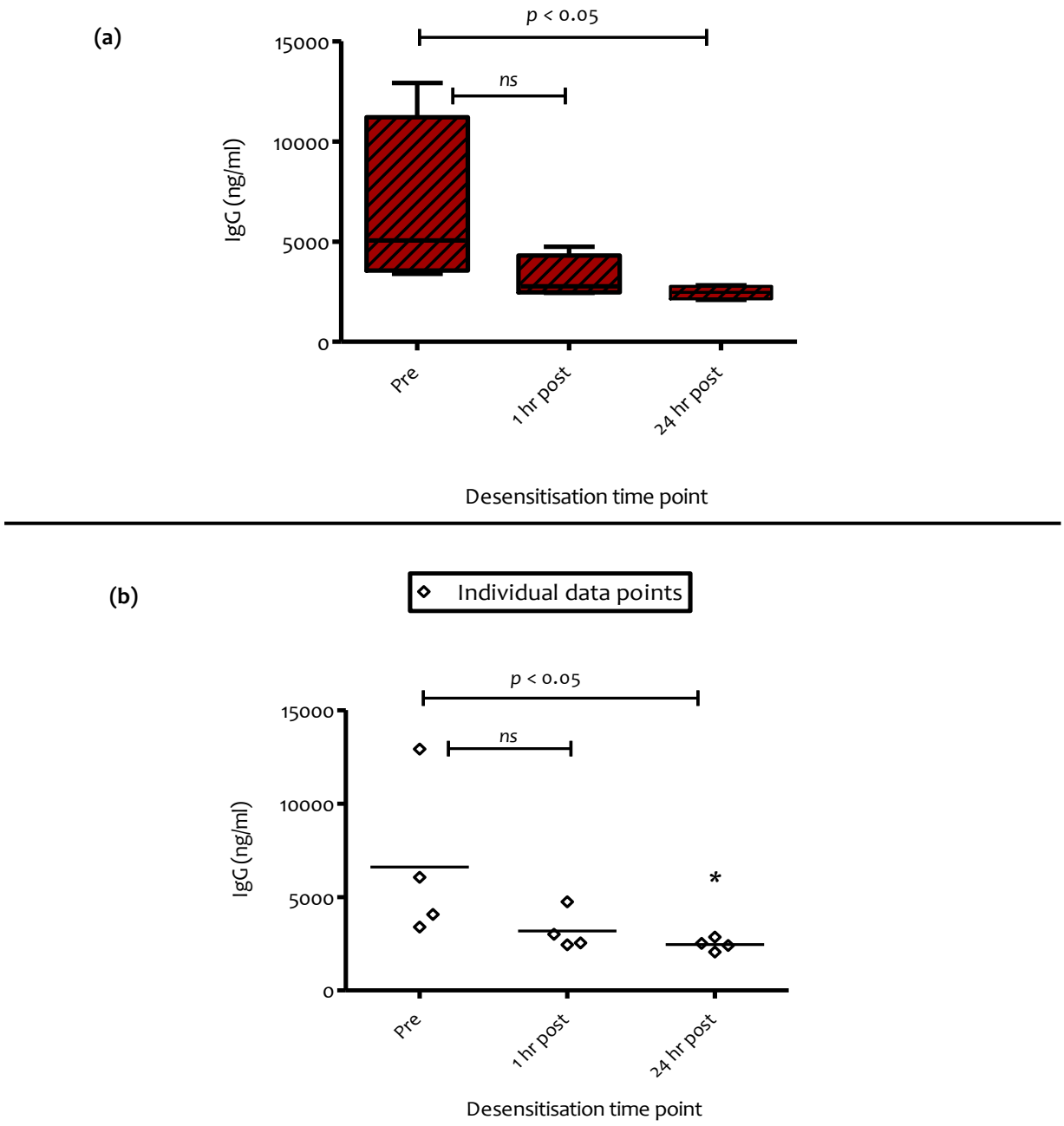


Figure 5.11 Analysis of the total IgG level of four desensitisation patient samples. Total IgG ELISA whisker plots (a) and dot plots (b) showing total IgG levels in patients (n = 4) prior to desensitisation and up to 24 hours post desensitisation to piperacillin. Data were analysed by the Students T-test to compare the difference between means with p≤0.05 considered as significant (****** denotes p = 0.001 to 0.009).

All samples were assessed for their ability to produce antidrug antibodies at the different time points during the course of piperacillin administration. Box plots show there is no significant difference between the amounts of anti-piperacillin antibody produced at all

the time points. One patient showed an extremely high level of anti-piperacillin-specific antibody which resulted in the large standard error exhibited by that group (the reaction group), See figures 5.12a and 5.12b.

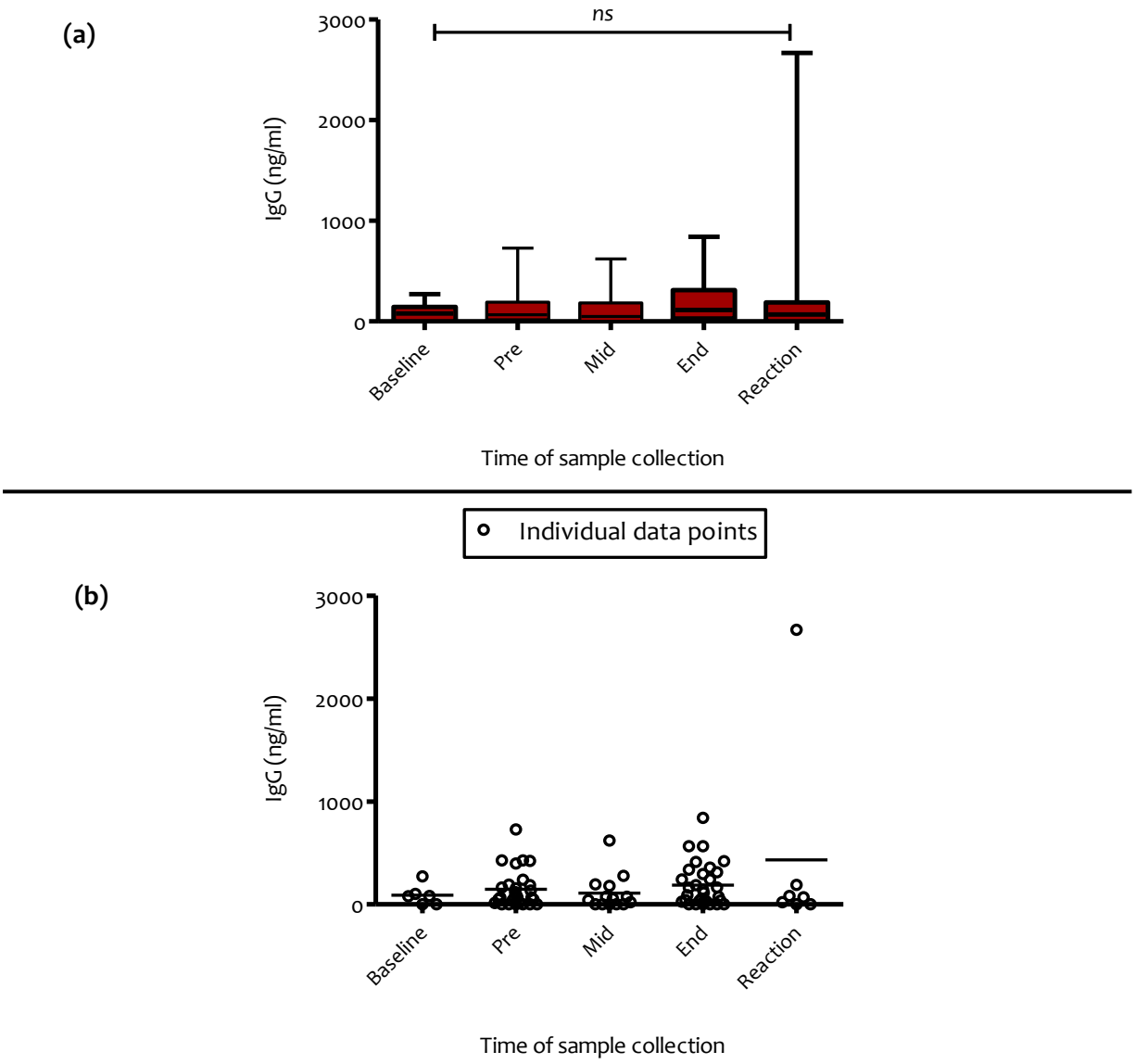


Figure 5.12 Kinetics of piperacillin-specific IgG detection in CF patients receiving piperacillin using ELISA. Whisker (a) and dot plot (b) of antipiperacillin antibody levels (ng/ml) in piperacillin treated patients with CF obtained at different stages of drug administration.

Further assessment was carried out on the kinetics of anti-piperacillin antibodies in four (4) hypersensitive patients pre- and 24h after the commencement of desensitisation. Plasma samples collected pre-desensitisation and 24 hours post-desensitisation were assessed for piperacillin-specific IgG using a hapten inhibition ELISA. The results indicated that at 24h post-desensitisation there was a significant reduction in the levels of circulating anti-piperacillin antibodies (figure 5.13).

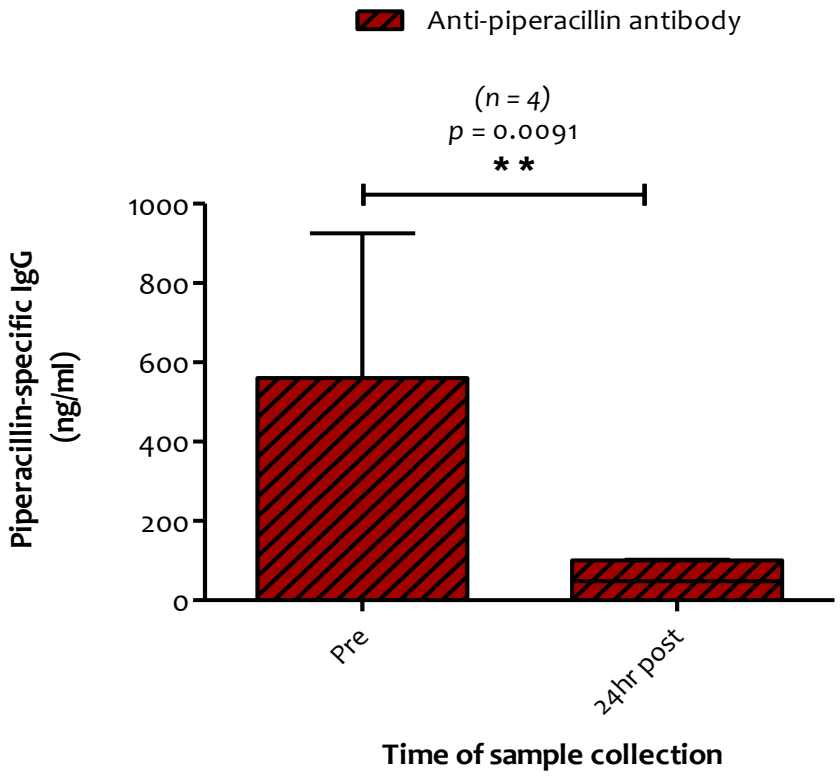


Figure 5.13 Time-dependent secretion of antidrug antibodies during piperacillin desensitisation. Plasma samples were obtained from patients undergoing piperacillin desensitisation (n=4). Samples were obtained pre-desensitisation and 24h after the start of desensitisation. A hapten inhibition ELISA was performed. Data were analysed by the Students T test to compare the difference between means with p≤0.05 considered as statistically significant. ** denotes *p* = 0.001 to 0.009.

5.3 Discussion

T-cells have been shown to proliferate in response to stimulation by drug antigens using the established lymphocyte transformation test (Luque, Leyva et al. 2001, Pichler and Tilch 2004). As I have stated previously in chapters 2, 3, and 4, LTT positive patients were considered as hypersensitive. This test was employed to establish when piperacillin-specific T-cells could be detected in the cohort of CF patients undergoing piperacillin therapy. A hypersensitivity screen of the individual patient drug courses (figure 5.1) and individual samples collected (figure 5.2) showed that the diagnosis of hypersensitivity was not restricted to a specific stage of drug treatment as positive LTT results were obtained from samples obtained prior to a specific course of piperacillin administration, during drug administration and at the onset of drug hypersensitivity when symptoms began to manifest (figure 5.2). Piperacillin-specific T-cell responses were also shown to persist retrospectively. Also hypersensitivity was detected in patients as early as their first course of treatment (n = 12) and second course (n = 5) as represented in figure 5.1. The detection of positive LTT is due to the fact that many patients have been exposed to prior courses of piperacillin. It also infers that there isn't a strict paradigm with respect to the number of courses required to facilitate the onset of hypersensitivity.

Two important points must be highlighted from this initial analysis of T-cell responses during the prospective study. First, these data identify positive LTT results in a small number of patients currently classified clinically as piperacillin tolerant. It will be interesting to see whether these patients develop a reaction when next exposed to piperacillin. Secondly, the negative LTT results obtained from certain acute allergy samples does not necessarily indicate an incorrect clinical diagnosis. A retrospective

sample still needs to be obtained from certain patients, whilst it is also possible that certain allergic patients present with a negative LTT. A significant difference in piperacillin-specific IgG was observed between LTT positive and negative samples (figure 5.5). This is the first indication of a relationship between the humoral and cellular components in piperacillin hypersensitivity. Further studies will be required to determine the relationship of these parameters to the clinical outcome.

The next approach involved assessment of the total IgG and piperacillin-specific IgG from baseline, pre, mid, end and reaction stages during piperacillin therapy. Studies have shown that the antibody type, and level detected during immune responses against infectious organisms is dependent on the organism and body/tissue compartment from which the samples analysed are collected. Differential expression of IgA, IgG and IgM antibodies has been observed in serum, bronchoalveolar lavage fluid, nasal secretions and saliva (Maiz, Cuevas et al. 2008, Aanaes, Johansen et al. 2013, Petrova, Strateva et al. 2013). An increase in certain specific antibodies to *Pseudomonas aeruginosa* and *Aspergillus fumigatus* in CF has been attempted previously but no mention was made of the total IgG levels (Pressler, Pedersen et al. 1992, Skov, Pressler et al. 1999). Tailoring our expectations along those lines would not be ideal simply because the nature of inflammatory immune response initiation and perpetuation of the immune system by infectious and non-infectious agents are fundamentally different (Kaczmarek, Vandenabeele et al. 2013). Subsequently using patient plasma we observed that there was no significant difference in total IgG levels between the various time points (pre, mid, end and reaction) during the course of piperacillin therapy (figure 5.7). Antidrug antibody levels also remained constant (figure 5.11a and 5.11b). Nonetheless, the

investigation of these levels in other body compartments should not be discountenanced as this may reveal a differential expression of clinical significance.

Drug desensitisation is routinely carried out in antibiotic hypersensitive patients with CF. The primary aim is to retain patients on preferred first line therapy, even in the presence of extreme disease conditions such as the severe decrease in lung function in CF patients. There are no formalised dosage regimens, but there have been a number of published reports recognising the validity of this process (Borish, Tamir et al. 1987, Cash, Caulder et al. 2013, Yusin, Klaustermeyer et al. 2013). The results obtained have been highly variable ranging from 24 % to 100 % success rates observed (Burrows, Toon et al. 2003, Turvey, Cronin et al. 2004, Legere, Palis et al. 2009, Whitaker, Naisbitt et al. 2012). Patients also require multiple desensitisations, prior to each course of the drug. In contrast to total IgG levels during piperacillin therapy, the desensitisation of hypersensitive patients showed a reduction of total IgG 1h after the start of desensitisation (see figure 5.9). This reduction was significant at 24h and with circulating piperacillin-specific antibodies 24 hours post-desensitisation. This is an indication that during desensitisation there is also a reduction in the total amount of circulating antibodies. The immuno-molecular mechanisms of successful desensitization have yet remained unclear and further studies will have to be performed to unravel the relevance of this on the clinical outcome therein.

In summary our initial screen of the first prospective study of drug hypersensitivity has established a few cardinal points:

- The LTT proved its usefulness in the experimental diagnosis of piperacillin hypersensitivity. Confirmation from clinical data will be required as some patients clinically diagnosed as hypersensitive did not develop drug-specific T-cell

responses. As stated earlier in this chapter, this may be due to misdiagnosis, a test deficiency or the absence of a retrospective sample and this can only be validated after a rechallenge with the drug.

- A relationship exists between piperacillin-specific T-cell responses and piperacillin-specific antibody levels during desensitisation. Further studies need to be carried out to explore the relevance of this relationship. Questions should also involve determining if this phenomenon is restricted to piperacillin hypersensitivity or applicable to drugs in general.
- Due to the chronic management of these patients with relatively high doses of β-lactams, most of the patients showed the presence of low levels of antidrug antibodies specific to piperacillin even in the absence of hypersensitivity. Thus, the presence of antidrug antibodies alone isn't an indication of hypersensitivity occurring, even though a reduction in the amount of circulating piperacillin-specific antibodies in hypersensitive patients is noted during desensitisation.
- Finally, there are no set time points during which these reactions arise though the data suggests a preference for the first and second courses of piperacillin administration.

Thus, with proper sample collection and patient recruitment the LTT has shown its potential relevance in the determination of the onset of piperacillin-specific T-cell responses as a precursor to the manifestation of clinical hypersensitivity. However, this conclusion is dependent on the reconciliation of patients' experimental and clinical data in order to generate a clearer picture.

CHAPTER 6

THE EFFECT OF IgG ANTIBODIES ON THE ACTIVATION OF DRUG-SPECIFIC T-CELLS FROM PIPERACILLIN HYPERSENSITIVE PATIENTS WITH CYSTIC FIBROSIS.

Table of Contents

Figures

Tables

6.0 Introduction

The humoral immune pathway classically terminates with the secretion of antibodies derived from a culmination of activities primary of which are B and T cell activation. The activation of B cells, due to biological or chemical agents could be T cell independent or dependent. T cell dependent responses have also been shown to play a role in the generation of antidrug antibodies (Jawa, Cousens et al. 2013).

While the role of T-cells in B-cell activation and differentiation leading to the development of the B cell response has been clearly elucidated, the same cannot be said of the role B cells and secreted antibodies play in the proliferation of T cells, and subsequent development of the T-cell response. Generally B cells have an established capacity for antigen presentation (Rodriguez-Pinto 2005, Chen and Jensen 2008) even though they are less potent in this regard when compared to dendritic cells (Corinti, Medaglini et al. 2000). While earlier studies have suggested antibody regulation of immune expression via the enhancement of antigen presentation (Celis, Zurawski et al. 1984), the potential of B cells and their secretions to have a pronounced effect on T cell function via other means should also not be disregarded. This is partly due to the presence of co-stimulatory molecules (Gimmi, Freeman et al. 1991, Grewal and Flavell 1996) and the B cell capacity to also secrete factors with known regulatory (Taga and Tosato 1992, Pistoia 1997, Moore, de Waal Malefyt et al. 2001, Anderton and Fillatreau 2008) and inflammatory functions (Locksley, Killeen et al. 2001, Ireland and Monson 2011).

Immunoglobulin G (IgG) is the most abundant immunoglobulin isotype present in human serum. The Fc portion of the IgG molecule (see figure 8, chapter 1) contains binding sites

for complement (C1q), IgG-Fc receptors (FcγR) on effector cells and neonatal Fc receptor (FcRn). The subclasses of IgG which include IgG1, IgG2, IgG3 and IgG4 share a 90% homology but still possess unique profiles. The individuality of these subclasses with respect to effector function exists mainly due to structural variations in the hinge regions (Hamilton 1987) and amino acid differences found in the N-terminal CH2 domain though other factors have been postulated (Hovenden, Hubbard et al. 2013, Theo and Gestur 2014). These variations give rise to the differential binding of IgG subclasses to the C1q and FcγR sites which in turn also determines the elicitation of a number of effector functions.

Table 6.1 Properties of Human IgG subclasses. Adapted from (Vidarsson, Dekkers et al. 2014)

	IgG1	**IgG2**	**IgG3**	**IgG4**
Molecular mass	146	146	170	146
Amino acids in hinge region	15	12	62[a]	12
Inter-heavy chain disulphide bonds	2	4[b]	11[a]	2
Susceptibility to proteolytic enzymes	++	+/-	+++	+
Mean adult serum level (g/l)	6.98	3.8	0.51	0.56
Proportion of total IgG (%)	60	32	4	4
Half-life (days)	21	21	7/~21[a]	21
Placental response	++++	++	++	+++
Complement activation				

Notes: [a] Depends on allotype [b] For A2 isomer

The effect of immunoglobulins on T-cell responses is exemplified through the use of purified intravenous immunoglobulin G (IVIG) *in vitro* with isolated PBMCs and CD3+ T cell populations (Lee, Koh et al. 2001, MacMillan, Lee et al. 2009). IVIG pooled from

multiple volunteers has been shown to impede immune cell effector functions, such as the activation and proliferation of T-cells and T-cell dependent polyclonal immunoglobulin production by B lymphocytes. The mechanisms underlying these effects are rather unclear with some groups suggesting an indirect mechanism via ancillary cells while others have suggested a direct T cell interference (Kondo, Ozawa et al. 1991, Bayry, Lacroix-Desmazes et al. 2003, Tha-In, Metselaar et al. 2007, Heidt, Roelen et al. 2009). Existing data shows that secreted immunoglobulins exert a certain amount of influence on the immune process. Thus, based on this current hypothesis we aimed in this chapter to characterize the regulatory activity of the antidrug antibodies on T cells from a piperacillin hypersensitive patient. Initially we attempted to generate B-cell lines secreting piperacillin-specific IgG (see chapter 3) to compare their antigen presenting capacity against IgG negative B cell lines and their influence on the nature of the induced T-cell response. Since the piperacillin-specific IgG secreting lines were not generated, an alternative strategy was adopted. Specifically piperacillin-responsive T-cell lines were cultured with drug and antigen presenting cells in the presence and absence of various plasma some of which contained piperacillin-specific IgG.

6.1 Methods

The methods employed include T cell proliferation assays, the ELISpot assay, and FACS analysis as illustrated in sections 2.1.9, 2.2.1, and 2.2.2.2 of the second chapter of this thesis.

6.2 Results

6.2.1 Quantification of antidrug antibodies in piperacillin hypersensitive patient plasma

The hapten inhibition assay (as previously described in chapter 2) was performed on plasma from two piperacillin hypersensitive patients. The amount of anti-piperacillin antibody which is the difference in antibody detection between the haptenated protein adduct (BSA:Pip) and the hapten inhibited (BSA:Pip + pip) samples was then quantified with antibody concentrations of 34.73 ng/ml and 28.80 ng/ml observed for patients 1 and 2 respectively (Figure 6.1).

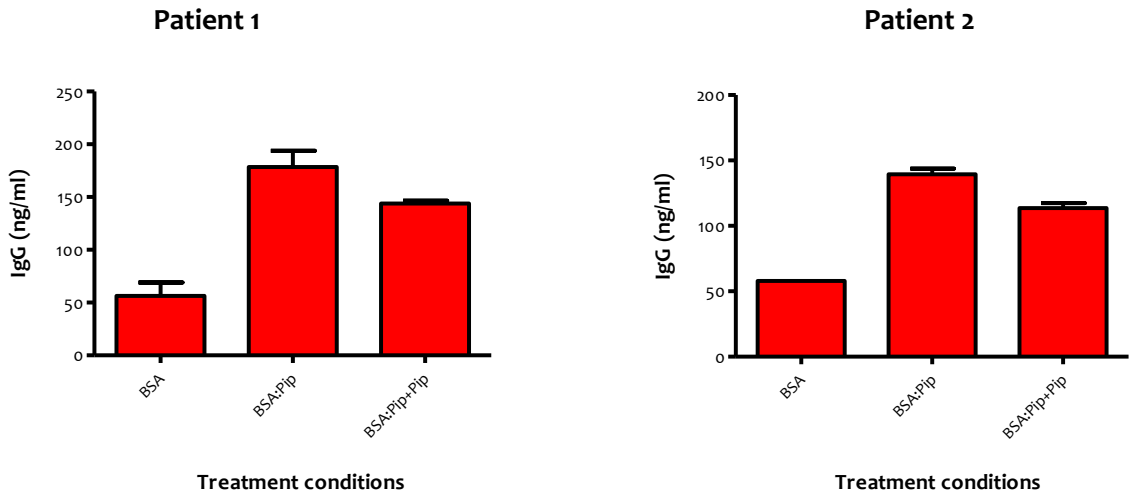


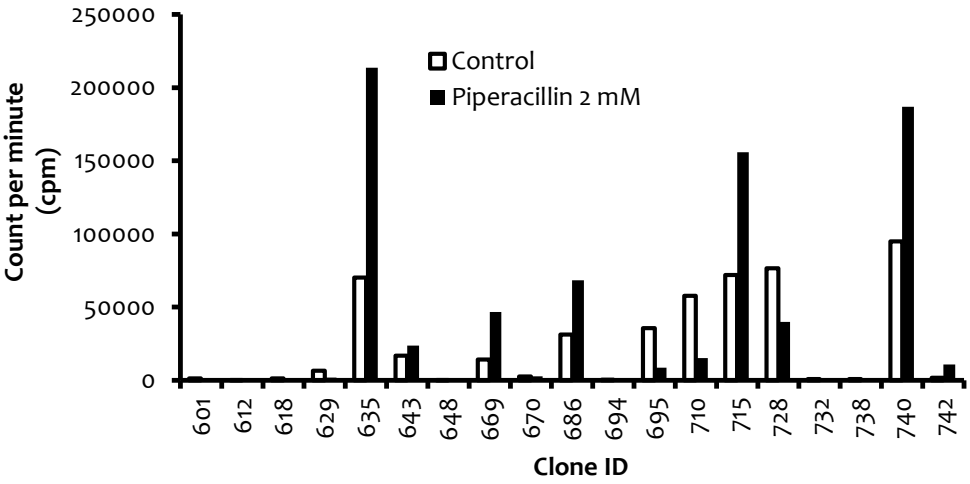
Figure 6.1. Detection of piperacillin-specific antibodies in hypersensitive patients. Graphs showing hapten inhibition assays carried out on 2 hypersensitive patient samples. High binding flat bottomed 96 well plates were pre-coated with 20 µg BSA:piperacillin adduct employed as an antigen. After washing, 100 µl of hypersensitive patient plasma with and without prior incubation with the hapten was added to wells and an ELISA carried out. Absorbance was read with an automated plate reader (Dynatech MR600).

6.2.2 Proliferation assay to determine piperacillin-specificity of T-cell clones

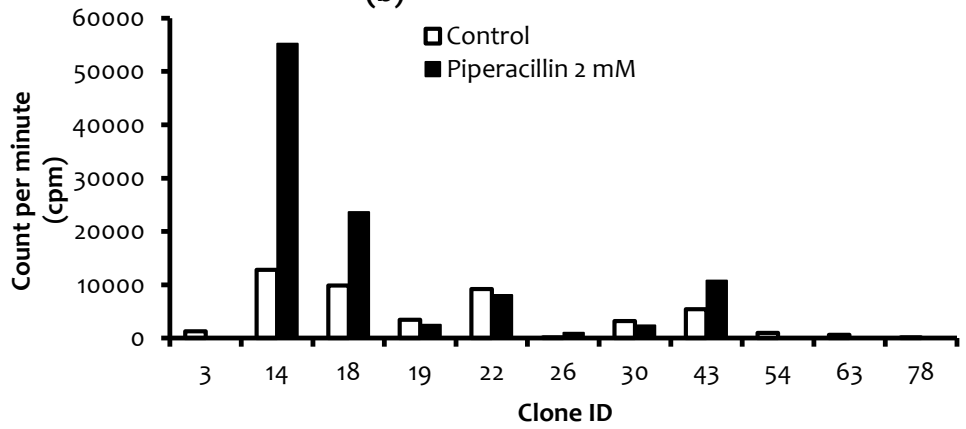
Nineteen T-cell clones were generated from hypersensitive patient 1 and eleven clones from patient 2, comprising twenty clones in total from both hypersensitive patients.

T cell clones from hypersensitive patients 1 and 2 were seeded in duplicate at concentration of 50 x 10^4 cells/well (100 µl) in a u-bottomed 96 well plate, with varying drug concentrations in R9 medium. To each of these wells of 1.0 x 10^4 autologous irradiated EBV transformed B-cells were added as a source of antigen presenting cells. The plates were then incubated at 37^{0}C and 5% CO_2 for 48 h. At the last 16 h of culture, cells were pulsed with [^{3}H] thymidine and plates subsequently counted using a beta counter. Eight clones from patient 1 and 3 clones from patient 2 were identified as being drug specific (figure 6.2a and 6.2b). The maximal proliferative responses seen over a range of doses for the different drug specific clones was assessed and graphs plotted (Figure 6.3a and 6.3b).

(a) **PATIENT 1.**



(b) **PATIENT 2.**



(c)

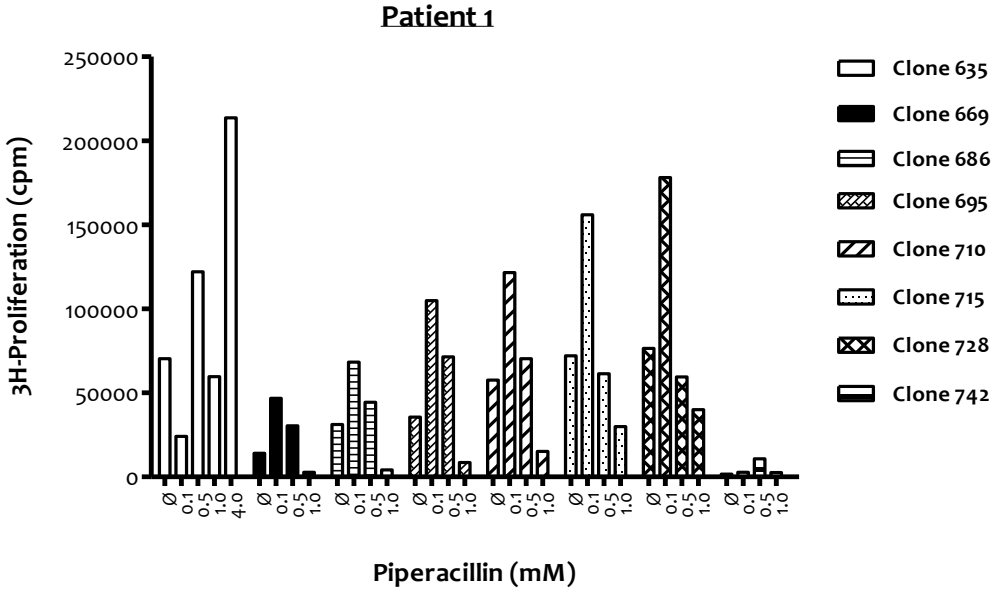
Patient ID	Clones tested	Pip specific clones	CD4+ (%)	CD8+ (%)
Patient 1	19	8	92%	8%
Patient 2	11	3	70%	30%

(a)

Figure 6.2

Proliferation assay to determine piperacillin-specificity of T-cell clones.

PBMCs (1.5×10^5 cells in 100 µL) from hypersensitive patient 1 (a) and 2 (b) were incubated with graded concentrations of piperacillin (0.125-4 mM in 100 µL) in 96-well U-bottom plates. Plates were incubated at 37°C under an atmosphere of 5% CO_2 for 48 hours. [3H]-thymidine (0.5 µCi/well) was added for the final 16 hours of incubation and T-cell proliferation measured using scintillation counting in a Beta counter. The data was analysed and a stimulation of index (SI) ≥ 2 considered as an indicator for specificity. (c) Table of the total number of T-cell clones generated from both patients.



(b)

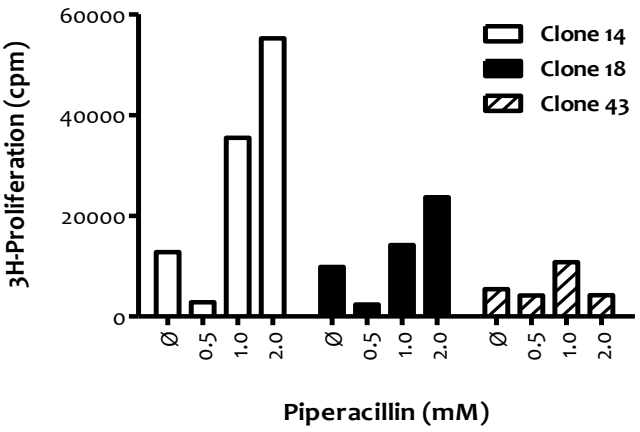


Figure 6.3

Dose response assay for piperacillin-responsive T-cell clones.

PBMCs (1.5×10^5 cells in 100 µL) from hypersensitive patient 1 (a) and 2 (b) were incubated with graded concentrations of piperacillin (0.1-4 mM in 100 µL) in 96-well U-bottom plates. Plates were incubated at 37°C under an atmosphere of 5% CO_2 for 48 hours. [3H]-thymidine (0.5 µCi/well) was added for the final 16 hours of incubation and T-cell proliferation measured using scintillation counting in a Beta counter. The data was analysed and a stimulation of index (SI) ≥ 2 considered as an indicator for specificity. (c) Table of the total number of T cell clones generated from both patients.

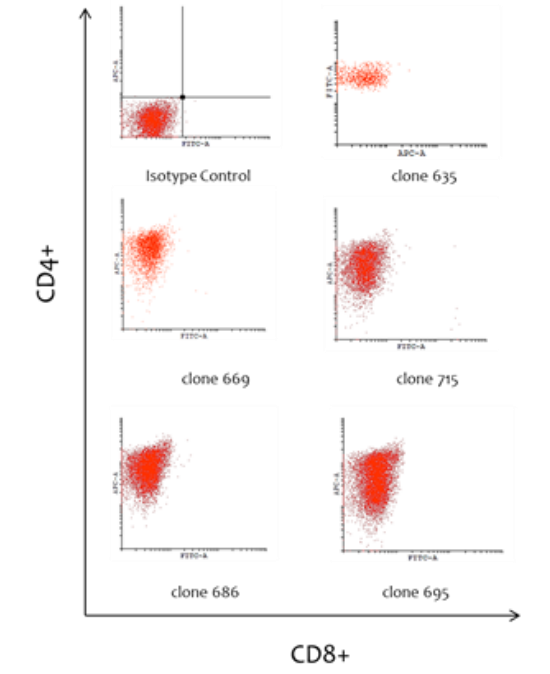
(c)

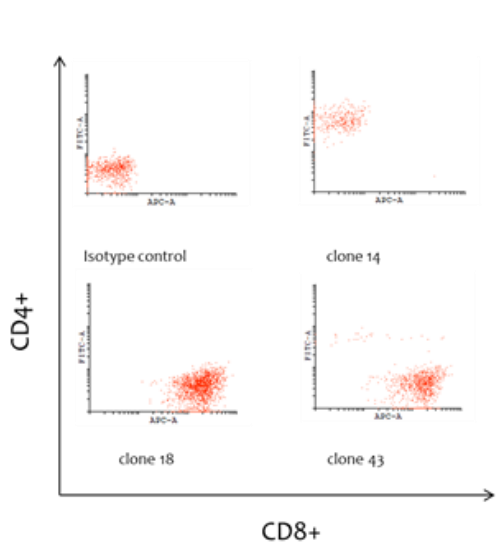
Patient ID	Pip specific clones	CD4+ (%)	CD8+ (%)
Patient 1	8	100%	—
Patient 2	3	100%	—

6.2.3 CD4+ and CD8+ phenotyping of piperacillin-specific T-cell clones

After assessing the piperacillin-specificity of the various T cell clones obtained from hypersensitive patients, we proceeded to identify the phenotype expressed by clones using flow cytometry analysis. Each T cell clone confirmed to be piperacillin-specific was counted and ≤ 5 x 10^5 cells of the T cell clone split equally and transferred into two FACS tubes. Cells were then stained with 3 µl of CD4 and CD8 fluorochrome antibodies, in the dark. After incubation at 4°C for 20 minutes, the tubes were washed with 1 ml FACS buffer and the supernatant discarded. The samples were then either re-suspended in 200µl of FACS buffer and the samples read on a FACS canto II flow cytometer. From the 11 clones tested 9 (73 %) were CD4+, 2 (27 %) were CD8+ (Figure 6.4).

(i)





(ii)

	Phenotype	Number
T cell lines from lymphocytes (18)	CD4+	8
	CD8+	–
	CD4+CD8+	–

	Phenotype	Number
T cell lines from lymphocytes (10)	CD4+	1
	CD8+	2
	CD4+CD8+	–

(iii)

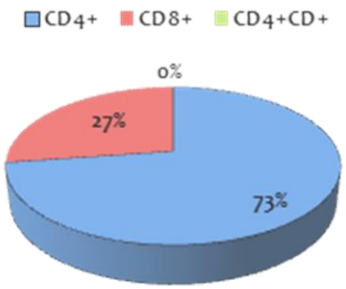


Figure 6.4. Flow cytometry analysis of piperacillin-specific T-cell clones. T cell clones were generated from 2 patients and stained with CD4 and CD8 fluorochrome antibodies to determine the phenotype (i) shows a dot plot representation of some piperacillin-specific T-cell clone phenotypes from patients 1 and 2 (ii) Table of the total number of piperacillin-specific T cell clones generated from both patients (iii) graph of the CD4+, CD8+ and CD4CD8 proportion of T-cell clones from the total number of clones generated from both patients.

6.2.4 Cytokine secretory profile of T-cell clones

Coating antibodies for the anti-cytokine antibodies were diluted in sterile PBS, pH 7.4. Prior to the addition of these antibodies, the PVDF membrane of the ELISpot plates were pre-wet with ethanol and then washed using Hanks balanced salt solution (HBSS) as described in materials and methods. Following the addition and overnight incubation of the required coating antibody solution at 4-8°C, 1.5 x 10^5 piperacillin-specific T-cell clones with 5 x 10^4 autologous EBVs alone, and with 2 mM piperacillin. 5 µg/ml PHA plates were incubated at 37°C and 5% CO_2 for 48 h in foil paper. The detection antibodies for T cell cytokines and a streptavidin-ALP (1:1000) substrate was employed for the development of the plates. Six clones were found to secrete IL-5, GB, IL-13 and IFN-γ. Following stimulation with piperacillin five clones (635, 669, 695,728 and 742) secreted IL-5. Granzyme B was not secreted from drug-stimulated clones. In addition to IL-5, clone 742 expressed high amounts of IL-13. IFN-γ was expressed by all the clones, though to different extents. A high level of IFN-γ expression was seen with clones 635, 669 and 695 while a lower expression was seen with clones 686, 728 and 742 (Figure 6.4).

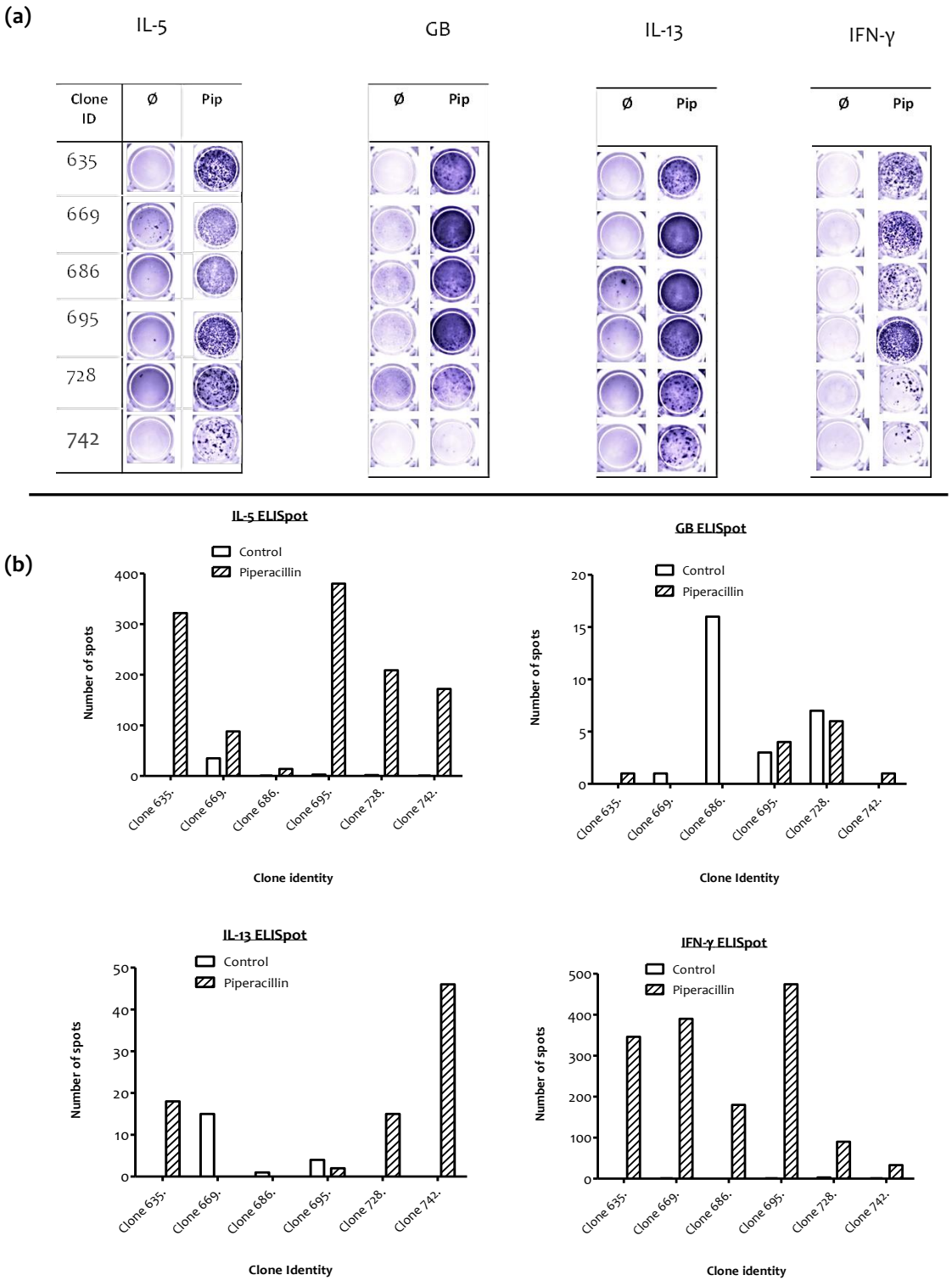


Figure 6.5 ELISpot Cytokine secretory profiles of hypersensitive patient 1. ELISpot plates were pre-coated with anti-IL-5, anti-GB, anti-IL-13, and anti-IFN-γ according to the manufacturers' instructions and incubated overnight at 4°C. Cells were harvested and transferred to each well and incubated for 48 h. ELISpot plates were developed according to the manufacturers' instructions and the wells left to air dry. Data were analysed with images captured using an AID ELISpot machine (a) and graphs plotted (b).

6.2.5 Proliferation assay to define the effect of antidrug antibodies on the piperacillin induced proliferative response of drug-specific T-cells

Three T cell clones (clones 669, 715, 742) from patients 1 and 2 were selected for the experiment. Clones (1.5 X 10^5) were cultured with autologous irradiated EBVs (5 x 10^4) and piperacillin in the presence of 1, 10 and 20 µl of plasma bearing anti-piperacillin antibodies from hypersensitive patients to corresponding wells to finally achieve a total well volume of 200 µl. The plates were then incubated at 37^{0}C and 5% CO_2 for 48 h. Proliferative responses were measured by the addition of [3H] tritriated thymidine for the final 16 h of the experiment. In the absence of autologous plasma piperacillin activated the clones with concentrations ranging 0.1 mM to 1 mM of piperacillin.

Autologous plasma was shown to inhibit the proliferative responses of T cells in a concentration-dependent fashion with a clone tested with at least one concentration of the drug. The results reached statistical significance with 1 mM piperacillin and 20 µl of plasma (p = 0.0307) [figure 6.6a]. With one clone (clone 742) inhibition of piperacillin induced T cell proliferation was observed at the lower drug concentration of 0.01 mM rather than at the higher dose of 1 mM as observed with the other two clones (figure 6.6c). The grouped data indicates a significant reduction in T cell proliferation with groups in which 20 µl of anti-piperacillin antibody bearing plasma were incorporated ($p \leq$ 0.05) [figure 6.6d].

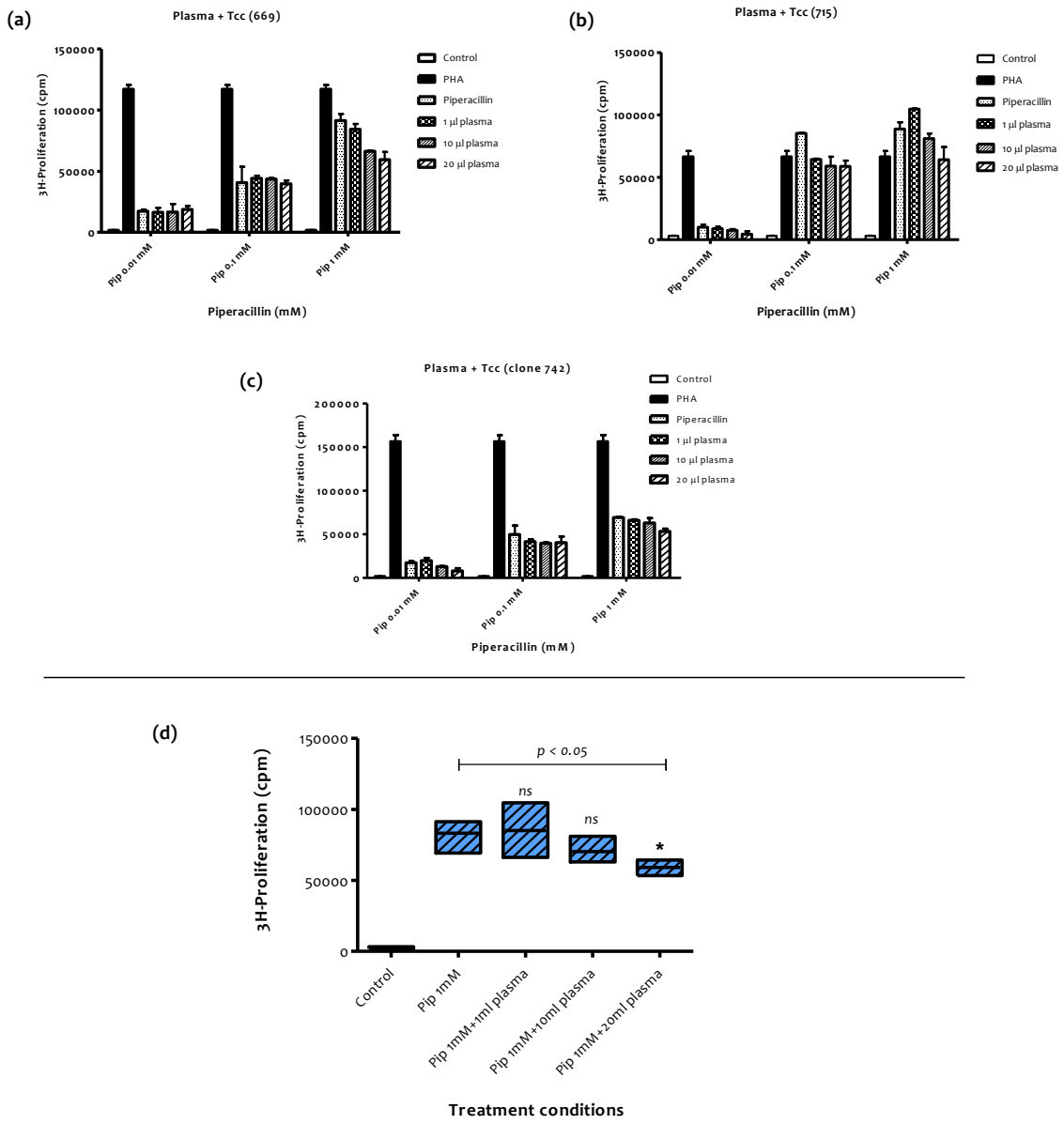


Figure 6.6 Effect of antidrug antibody bearing plasma on the T-cell proliferative responses. Three piperacillin-specific T-cell clones (1.5×10^5 cells in 100 µL) from hypersensitive patient 1 (a, b and c) were incubated with graded concentrations of piperacillin (0.01 – 1.0 mM in 100 µL) and anti-piperacillin antibody bearing plasma (0.01 – 20 µL in 100 µL) in 96-well U-bottom plates. Plates were incubated at 37°C under an atmosphere of 5% CO_2 for 48 h. [3H]-thymidine (0.5 µCi/well) was added for the final 16 h of incubation and T-cell proliferation measured using scintillation counting in a Beta counter. The data were analysed by Students T test with $p < 0.05$ considered significant (***** denotes $p = 0.05$ to 0.01) (d) Proliferative responses at 1 mM of three piperacillin-specific T-cell clones (1.5×10^5 cells in 100 µL) from hypersensitive patient 1 were analysed by Students T test with $p < 0.05$ considered significant (***** denotes $p = 0.05$ to 0.01).

In subsequent experiments, 1.5 x 10^5 piperacillin – specific T cell clones (635, 686 from patient 1 and 14 from patient 2) were cultured with autologous irradiated EBVs (5 x 10^4) and piperacillin in the presence of 20 µl of autologous plasma from naïve volunteers added to corresponding wells to finally achieve a total well volume of 200 µl. The plates were then incubated at 37°C and 5% CO_2 for 48 h. Proliferative responses were measured by the addition of thymidine for the final 16 h of the experiment. A significant attenuation of drug-specific T cell clone proliferation due to 1 mM piperacillin in the presence of plasma was also observed (figure 6.7a, b and c).

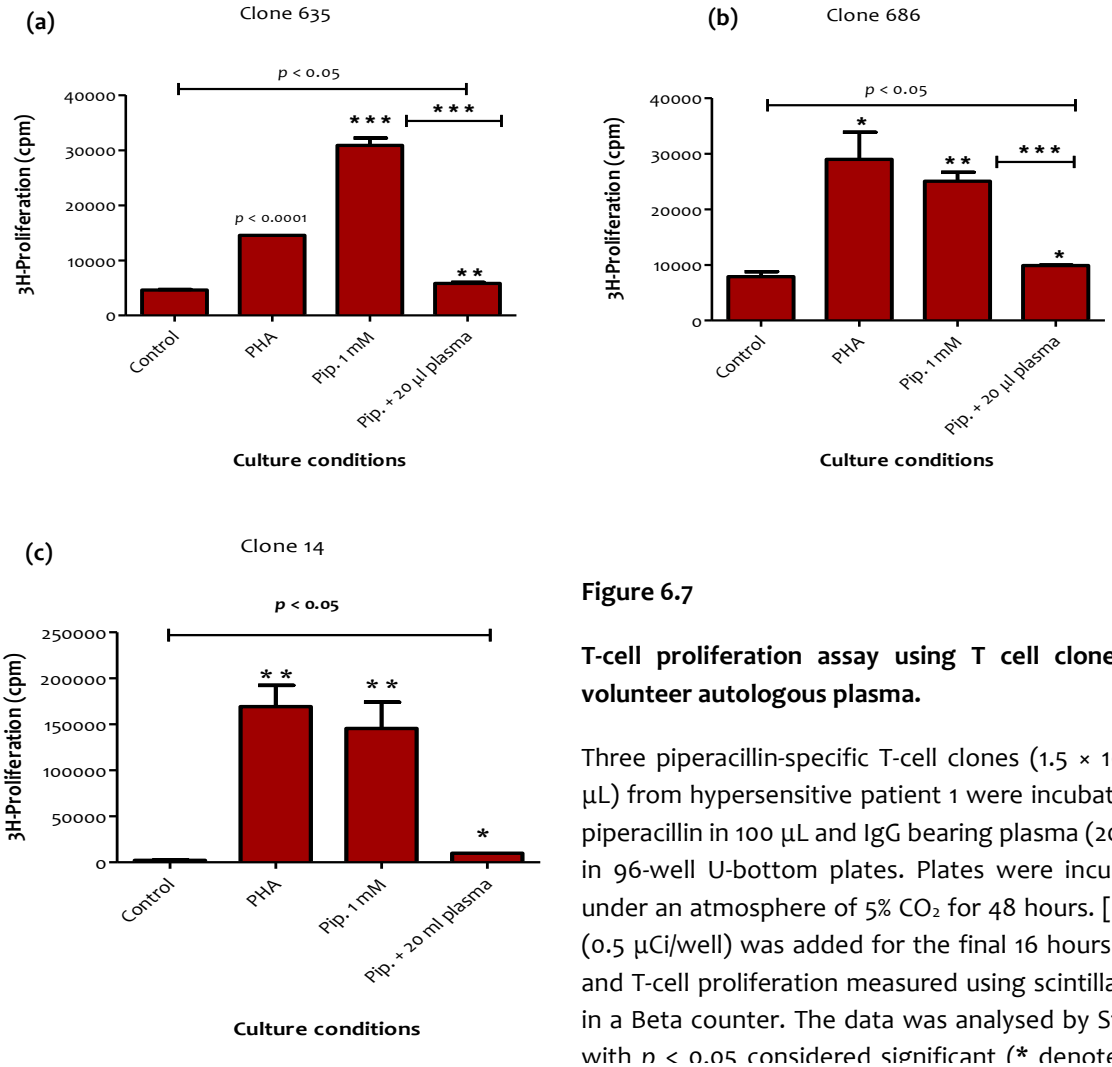


Figure 6.7

T-cell proliferation assay using T cell clones with naïve volunteer autologous plasma.

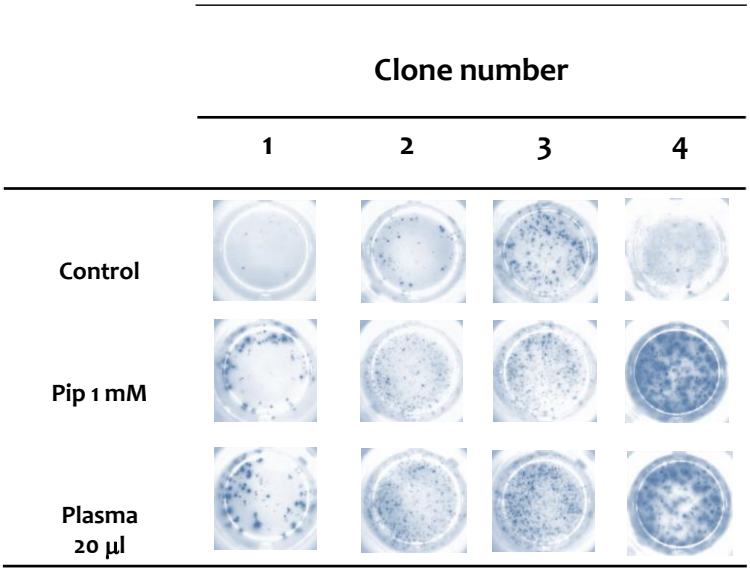
Three piperacillin-specific T-cell clones (1.5 × 10^5 cells in 100 µL) from hypersensitive patient 1 were incubated with 1 mM piperacillin in 100 µL and IgG bearing plasma (20 µL in 100 µL) in 96-well U-bottom plates. Plates were incubated at 37°C under an atmosphere of 5% CO_2 for 48 hours. [3H]-thymidine (0.5 µCi/well) was added for the final 16 hours of incubation and T-cell proliferation measured using scintillation counting in a Beta counter. The data was analysed by Students T-test with $p < 0.05$ considered significant (* denotes $p = 0.05$ to

6.2.6 ELISpot assay to define the effect of anti-piperacillin antibodies on the piperacillin induced IFN-γ secretion of drug-specific T-cells

Experiments were also carried out to determine the effect of plasma from hypersensitive patients and naïve volunteers on IFN-γ secretion from drug-specific clones.

Coating antibodies for the anti-cytokine antibody were diluted in sterile PBS, pH 7.4. Prior to the addition of the antibody, the PVDF membrane of the ELISpot plate was pre-wet with ethanol and then washed using Hanks balanced salt solution (HBSS) as described in materials and methods. Following the addition and overnight incubation of the required coating antibody solution at 4-8°C, 1.5 x 10^5 piperacillin-specific T-cell clones with 5 x 10^4 autologous EBVs alone, and with 2 mM piperacillin and 5 μg/ml PHA plates were incubated at 37°C and 5% CO_2 for 48 h. The detection antibody for IFN-γ and a streptavidin-ALP (1:1000) substrate was employed for the plate development. The wells left out to air dry and spots were visualised using an AID ELISpot reader. The incorporation of 20 μl naïve volunteer plasma in the T cell proliferation assay (n = 4) did not produce a reduction in the total IFN-γ secretion; depicted as number of spots in the wells, from drug-specific T cell clones when compared to the piperacillin wells alone (figure 6.8a). The introduction of 20 μl piperacillin hypersensitive patient plasma bearing antipiperacillin antibody on the other hand showed the attenuation of IFN-γ cytokine secretion with all but one clone (6.8b).

(a) Naïve volunteer plasma minus piperacillin-specific antibody.



(b) Hypersensitive patient plasma containing piperacillin-specific antibody

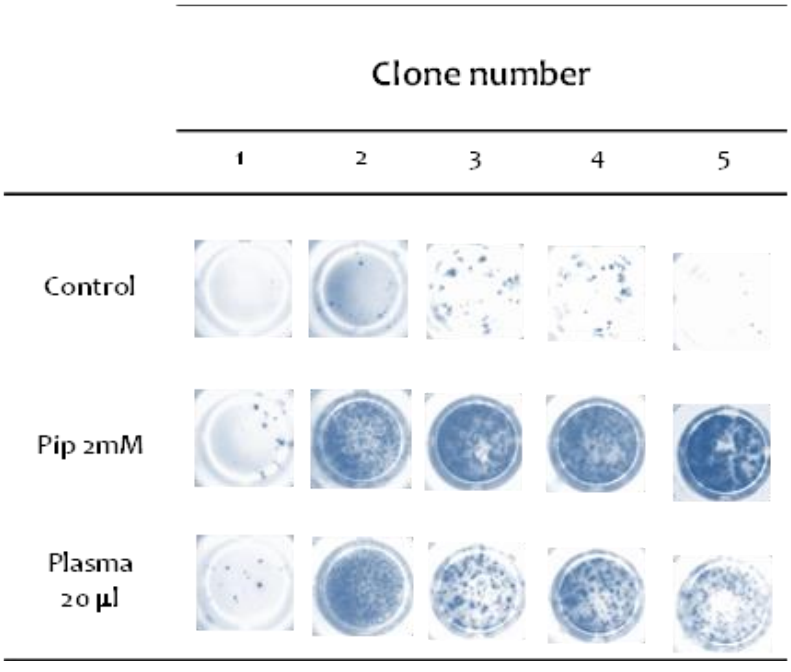


Figure 6.8 IFN-γ ELISpot using piperacillin-specific T-cell clones. ELISpot plates were pre-coated with anti-IFN-γ according to the manufacturers' instructions and incubated overnight at 4°C. Cells were harvested and transferred to each well and incubated for 48 h under the stipulated culture conditions with (a) naïve volunteer plasma and (b) hypersensitive patient plasma containing piperacillin-specific antibody. ELISpot plates were developed according to the manufacturers' instructions and the wells left to air dry. Data were analysed with images captured using an AID ELISpot machine.

6.3 Discussion

As stated in earlier chapters, B cells and secreted antibodies play a significant role in the immune response. Delayed hypersensitivity reactions in CF patients due to β-lactam administration, have been shown to involve antigen-specific T cells. However, the contribution of other cellular components of the immune system especially B-cells, immunoglobulins and antidrug antibodies to the iatrogenic disease has not been established.

High levels of antigen specific IgG4 antibodies have been found to be beneficial in specific immunotherapy (SIT). SIT is an immune-modifying therapy that has been recommended for the treatment of allergic rhinitis, venom hypersensitivity, some drug allergies and mild bronchial asthma. The induction of immunological tolerance and of blocking IgG4 antibodies through repeated exposure to allergen(s) being the major characteristics (Holgate and Polosa 2008).

Different attempts at explaining this association suggest that (i) high levels of IgG4 production is indicative of Treg activation culminating in the production of anti-inflammatory factors such as IL-10 and TGF-β; and (ii) IgG4 antibodies have the capacity to act as blocking antibodies. B cells have the ability to produce antibodies and cytokines such as IL-10 though the relationship between the production of these cytokines, antigen-specific IgG and the attenuation of hypersensitivity symptoms still remains poorly defined (Hagan, Blumenthal et al. 1991, Hussain, Poindexter et al. 1992, Aalberse and Platts-Mills 2004, Aalberse 2011).

Plasma containing immunoglobulins and also purified IVIG alone has been shown to inhibit the proliferation of T cells. Whole PBMCs have been employed in the analysis of these effects in spite of the inherent drawbacks of coexisting functional immune cells (Klaesson, Ringden et al. 1993, Lee, Koh et al. 2001). Thus, to eliminate the presence of auxiliary cellular components interfering with the T cell response to IVIG in a synergistic or otherwise manner, CD3+ cells purified from mononuclear cells have also been studied (Heidt, Roelen et al. 2009, Aubin, Lemieux et al. 2010) and similar inhibitory responses have been detected. Additionally the antibody concentration in plasma required to elicit that given inhibitory response has not been determined, though dose response assays have been carried out *in vitro* using IVIG (Aubin, Lemieux et al. 2010). I hoped that through this study we would be able to fill this gap.

Anti-piperacillin antibodies were detected and quantified in the plasma of piperacillin hypersensitive patients (see chapter 3) out of which two patients [see figure 6a (i) – (iv)] were selected. For the current study T-cell clones and EBV immortalized B cells were then generated from these two patients. Patients were selected from the cohort of hypersensitive patients that bore anti-piperacillin antibodies based on the ability to generate both immortalized B-cell lines and piperacillin-specific T cell clones. Drug-specific T-cell clones were generated from the hypersensitive patients and characterized in terms of cellular phenotype and function. All clones (both CD4 and CD8+) were stimulated to proliferate with piperacillin in a concentration-dependent manner.

Six clones (CD4+) from patient 1 secreted IFN-γ. The majority (5) of these clones also secreted IL-5. However, only one clone secreted IL-13. A larger proportion of clones from patient 2 were CD8+ when compared to patient 1, even though the Th1/Th2 archetype has

been used to explain the basis of different disease conditions (Wynn 2004, van Oosterhout and Motta 2005). Unfortunately, a large proportion of the T cell clones including all CD8+ clones obtained from this patient failed to proliferate properly after repeated re-stimulation which prevented their use in subsequent assays. For this reason the assays were largely based on Th2 secreting CD4+ T cell clones. If plasma from the patients blocked proliferative responses to piperacillin, these same clones would have been used to study cytokine release in the presence and absence of piperacillin-specific IgG.

The proliferative responses of these clones were then assessed utilizing EBV immortalized B cells as antigen processing cells and different amounts of autologous plasma bearing antidrug antibodies. The objective of these experiments was to establish a limit at which T cell proliferation could be blocked. The expectation was that a marked inhibition of T cell proliferation would have been observed at lower concentrations of plasma.

A significant reduction in T-cell proliferation was observed with all clones from the two patients with 20 µl of piperacillin-specific antibody bearing plasma. This translated to concentrations of approximately 3.43 pg/ µl in patient 1 and 2.88 pg/ µl in patient 2 of piperacillin-specific IgG which presented a uniform inhibition of proliferation. Lower concentrations of antibody had less of an inhibitory effect. Unexpectedly, this reduction was not specific to piperacillin antibody bearing plasma as 20 µl of plasma from naive volunteers also produced the same response. The use of whole plasma rather than purified drug-specific antibodies present the possibility that these non-specific protein interactions could have been responsible for the similarities in responses obtained.

Therefore using the ELISA to determine if the reduction could be due to the presence of specific-IgG or total IgG as a whole was difficult. Following piperacillin stimulation of piperacillin-specific T-cell clones there was an increased secretion of IFN-γ. The incorporation of naïve and piperacillin antibody bearing plasma produced varying levels of IFN-γ secretion from T cell clones. Naïve volunteer plasma did not affect the IFN-γ secretion from piperacillin-specific T cell clones, while the introduction of plasma bearing antipiperacillin antibody produced an attenuation of cytokine secretion with all but one clone used in the assay.

Though different chromatographic methods of purification of these antibodies using protein G Sepharose, protein A/G Sepharose, polyethylene glycol and caprylic acid-ammonium sulphate precipitation do exist (Bergmann-Leitner, Mease et al. 2008), time constraints made the purification of these antibodies impossible. The seeming inconsistency that exists between data that was generated from the proliferation and cytokine release assays suggests that more work would have to be carried out to actually define the roles played therein.

CHAPTER 7

FINAL DISCUSSION

Table of Contents

7.0 Final Discussion

Drug hypersensitivity reactions make up a significant proportion of ADRs and have been extensively studied in terms of the involvement of the immune system in the pathogenesis. Moreover, in recent years we are beginning to understand the chemical, pharmacological and genetic factors that contribute towards individual susceptibility (Pichler, Naisbitt et al. 2011). Cells of the innate and adaptive immune system and antibodies released by B cells (Antunez, Martin et al. 2006, Posadas and Pichler 2007, Bircher and Scherer 2010) are believed to be responsible for the elicitation of these reactions. T cells have been previously identified to play a significant role in the pathogenesis of these reactions, but the effect of B cells has yet to be defined (Whitaker, Naisbitt et al. 2012).

Hypersensitivity reactions were historically classified according to the time and onset of clinical symptoms; i.e., immediate or delayed hypersensitivity reactions. The importance of B cells can be viewed from the participation of these cells or their secretions in patients with delayed and immediate forms of hypersensitivity. Immediate hypersensitivity reactions primarily involve IgE-mediated release of histamine and other mediators of inflammation from mast cells and basophils. Delayed reactions on the other hand are essentially mediated by mononuclear leukocytes with cell components playing different but equally important roles of antigen presentation as seen with the B cells and dendritic cells, immune regulation and antigen killing as observed with CD4+ and CD8+ T cells (Rodriguez-Pinto 2005, Zhu, Yamane et al. 2010). In addition to their roles mediators of immediate hypersensitivity reactions, IgA and IgG, both of which are secreted by B lymphocytes, have been detected in certain patients with delayed hypersensitivity

(Vojdani, O'Bryan et al. 2008); however, their role in elicitation/regulation has not been fully defined.

Based on the roles played by B lymphocytes and their secretions an important objective of this thesis was to modify existing methods and eventually develop new methods to detect drug-specific B-cell antibody secretion using samples isolated from piperacillin tolerant and hypersensitive patients with CF. Piperacillin was selected for 2 reasons. First, it is a β-lactam antibiotic and the chemistry of antigen formation has been delineated. In fact, the amino acid residues modified by the drug hapten have been characterized on plasma albumin of patients exposed to a therapeutic treatment course (Whitaker, Meng et al. 2011). Secondly, piperacillin exposure is associated with a high frequency of hypersensitivity reactions in patients with CF (Koch, Hjelt et al. 1991, Wills, Henry et al. 1998, Burrows, Nissen et al. 2007); thus, clinical samples were readily available for functional studies. We also generated and characterized piperacillin-protein adducts for use as antigens to detect the presence of antibodies in the hypersensitive patients. Finally, the methods utilized enabled us explore, the B cell-mediated regulation of drug antigen-specific T cell proliferation.

An extensive search of existing literature describing the molecular basis of the immune response before, during and after the onset of hypersensitivity reactions provided us with a foundation on which to base our investigations. Our searches brought to fore the basic assays utilized in the confirmation of hypersensitivity (Nyfeler and Pichler 1997, Jurado-Palomo, Cabanas et al. 2010), and more complex characterization of cellular involvement with respect to the pathology and eventual clinical consequence (Gerber and Pichler 2004, Naisbitt, Farrell et al. 2005, Krumbholz, Pellkofer et al. 2007, Harrer,

Lang et al. 2010). From this literature review, a host of cell culture-, colorimetric- and proteomic-based assays were selected and applied to achieve the aims stipulated above.

In the first experimental chapter I focussed on the development and optimization of cell culture methods for the detection of specific B cell responses to piperacillin haptens using samples from hypersensitive and tolerant patients with CF. I also attempted to apply molecular methods to assess the presence or not of piperacillin-specific IgG and IgG subclasses in the same cohort of patients, while aiming to develop long-lived B cell lines equipped with the ability to secrete drug-specific antibodies. Our initial thoughts being that the development of these lines was essential in producing a B cell T cell co-culture system that closely mimics the *in vivo* situation to study the regulation of antigen-specific T cell responses by B cells.

Piperacillin hypersensitivity was confirmed in allergic patients using the lymphocyte transformation test. This test used [^{3H}] thymidine to detect the proliferation of T cells stimulated with mitogen or tetanus toxoid (positive controls), piperacillin and medium alone. An increase in incorporated radioactivity after a 6 day culture period in drug-treated wells is indicative of sensitization. The assay has been applied previously to diagnose a range of drug hypersensitivity reactions and a high sensitivity (positive response in hypersensitive patients) and specificity (negative response in tolerant controls) has been reported (Naisbitt, Nattrass et al. 2014). T-lymphocytes from piperacillin hypersensitive patients were stimulated to proliferate in a dose-dependent manner when cultured with the drug. In contrast, T-lymphocytes from tolerant controls were not specifically stimulated. Utilising similar methods, the phenotype of B-cells from tolerant and allergic patients was assessed in cultures in the presence and absence of

piperacillin. A significant increase in CD27 expression on CD19+ B cells was detected by flow cytometry when cells from hypersensitive patients were cultured with piperacillin. In contrast, an increase in CD27 expression was not detected on cells from tolerant controls. CD27 is a B-cell activation marker and its expression is up regulated upon IgG release. The increase in expression of CD19+CD27+ B cells in hypersensitive patient PBMC after drug stimulation *in vitro* gave a clear indication of the presence of immunological B-cell memory. Marked secretion of IgG by PBMCs in response to piperacillin treatment was also observed with hypersensitive patients using a B cell ELIspot that detects secreted IgG. B cells from naïve donors or tolerant patients were not stimulated to secrete IgG with piperacillin. Quantification of piperacillin-specific IgG and IgG subclasses was not achievable by ELISA using cell culture supernatants due to the small amounts of antibody present therein, but was performed on whole plasma isolated directly from patient blood. Piperacillin hapten-specific IgG was detected in significant quantities in hypersensitive patient plasma. A bias for IgG2 over other subclasses was also shown.

Three hundred and forty eight B-cell lines were generated from three hypersensitive patients. 10.05 % of the lines secreted IgG, but drug-specific IgG was not detected. Further attempts to modify the method of B cell line generation were attempted to explore the influence T cells have on the production of drug-specific antibodies *in vivo*. Specifically, T-cell cytokines were introduced to the B cell culture system in an attempt to influence B cell differentiation. This resulted in an increase in the efficiency of antibody production (99.2%) but similar outcomes with respect to drug-specific antibody production. Lines producing piperacillin hapten-specific IgG were not detected.

Despite the inability of our modified culture system to generate B cell lines secreting quantifiable amounts of drug-specific antibody we were able to confirm the validity of the cell culture and molecular methods used in the detection of B cell responses. In doing so we were able to establish a profile for the generation of piperacillin-specific IgG producing CD19+CD27+ cells. The absence of these cells in naïve patients and their marked visibility in hypersensitive patients being an initial pointer implicating the generation of these antibodies in the pathogenesis of piperacillin hypersensitivity. However, the roles they play in elicitation/regulation of the immune response and their influence on the proliferative capacity of T-cells still had to be characterized.

The second experimental chapter was primarily concerned with generation and characterization of β-lactam protein adducts and the influence of adduct structure on the propagation of antibody mediated B-cell responses in hypersensitive patients. The covalent binding of drugs or haptens, as reactive derivatives of drugs are often referred to as, to endogenous proteins has been established as a precursor to the onset of immune activation (Naisbitt, Gordon et al. 2000, Schnyder and Pichler 2009). The *in vitro* replication of this concept known as the hapten theory of drug hypersensitivity has focussed in recent years on T-cell immunogenicity. β-lactam-protein adducts have been generated and characterized to define the nature of the amino acid residue modifications which lead to development of epitopes for T-cells. Drug concentration and duration of exposure influence the extent of drug binding as well as the sites of modification and this impacts on whether or not a T-cell response is detected. In the current study HSA was initially used as a protein carrier however unconjugated HSA resulted in high backgrounds in ELISA assays to detect IgG. Other authors have experienced and

documented similar findings (Ming Kei Chung, Jacques Riby et al. 2010, Whitaker, Meng et al. 2011, Ariza A, Mayorga C et al. 2015). Thus, a suitable replacement to HSA that had a low background in the ELISA, but also possessing the requisite lysine residues to facilitate β-lactam binding was sought. Eventually BSA was selected. A clear relationship was observed between the additive increase in conjugate molar ratio to hapten density and subsequently to detection of specific IgG antibodies in patient plasma. The absence of cross reactivity with other β-lactam-BSA adducts highlighted the specificity of the antibodies to piperacillin the drug hapten the patients were exposed to at the time of the hypersensitivity reaction.

Even though I was successful in detecting the presence of piperacillin-specific IgG antibodies in plasma of hypersensitive patients, the role these antibodies play in the disease pathogenesis had not been explored. Hence, the next chapter utilized samples collected prospectively from patients exposed to multiple courses of piperacillin some of whom went on to develop an allergic reaction. The first priority was to characterize the CF patient cohort to assess the various trends including T cell proliferation, total and drug-specific antibody levels, and specific cytokine profiles before, during and after drug administration.

A few salient points were observed from our analysis of drug-specific T cell responses. First, most patients classified clinically as tolerant yielded reproducible negative lymphocyte transformation test results with piperacillin. Thus, the assay has a high specificity in this patient cohort. Secondly, piperacillin-specific lymphocyte proliferative responses were detected in the acute phase of certain clinically diagnosed hypersensitivity reactions. An important component of the clinical design was to

incorporate a retrospective blood sample at least 1 month after resolution of hypersensitivity. Positive lymphocyte transformation from 45% of hypersensitive patients was observed even if the acute sample yielded negative results. Since sample collection is on-going, several retrospective samples are still to be collected; hence, it is currently not possible to state the percentage lymphocyte transformation test positivity in the hypersensitive patient group. Thirdly, a small sub-set of tolerant patients showed positive lymphocyte transformation test results during the final course of piperacillin, but are still classified clinically as tolerant to the drug. Thus, it will be interesting to observe whether these patients develop a hypersensitivity reaction when next exposed to the drug. Fourthly, not all reactions clinically diagnosed as piperacillin hypersensitivity reactions were confirmed using the acute or retrospective lymphocyte transformation test. This may be because certain reactions are mediated via non-immune mechanisms (Bernstein 1995 , McKenna and Leiferman 2004). Alternatively, the negative result may relate to a misdiagnosis or a test deficiency. Finally, analysis of the drug-specific T-cell responses of individual patients exposed to repeated piperacillin courses revealed that there are no specific time-points during which reactions arise.

Significant differences in piperacillin-specific antibody levels were found to exist between lymphocyte transformation test positive and negative patients. Higher levels of piperacillin-specific IgG were detected in the lymphocyte transformation test positive samples. A small number of hypersensitive patients were recruited to the study as they were timetabled to undergo desensitisation (graded drug challenge). Following desensitisation most hypersensitive patients are able to tolerate a therapeutic course of piperacillin. Interestingly, lower levels of piperacillin-specific IgG were detected 24h post-desensitisation and each of these patients tolerated piperacillin. Finally, low levels of

piperacillin-specific antibodies were detectable irrespective of the presence or not of hypersensitivity diagnosis. This may be attributed to the long term management of these patients and exposure to repeated courses of piperacillin. To conclude, the presence of anti-drug antibodies alone does not seem to represent a diagnostic characteristic of hypersensitivity. However, their occurance in high levels does seem to relate to the development of a piperacillin-specific cellular immune response. Further studies need to be carried out to determine the clinical implications of this relationship.

The ability of intravenous antibody formulations, specifically IVIG to inhibit T cell proliferation has been established. This motivated us to explore whether plasma levels of circulating antibodies modulate piperacillin-specific T-cell responses *in vitro.* Classical proliferation assays were employed using piperacillin-specific T cell clones from hypersensitive patients in the presence or absence or culture medium supplemented with various amounts of antibody containing plasma. The ultimate aim being the appraisal of the resultant ability or inability as the case may be to inhibit the proliferation of T cells. The initial inference was that the assay was not sensitive enough as inhibition of proliferation though present occurred at seemingly high volumes of plasma with lower concentrations being unable to facilitate any inhibition. This was quashed to some extent due to the fact that subsequent quantification of drug-specific antibodies in patient plasma used in the assays showed concentrations of 3.43 pg/ml and 2.88 pg/ml for both patients. These data were one of the major driving forces prompting us to attempt to generate B-cell lines that secrete piperacillin-specific IgG. However, as we were not successful in generating lines that could be used as autologous antigen presenting cells alongside their ability to secrete IgG, the significance of the original observations remains somewhat speculative.

The fine differences that determine whether drug exposure leads to an allergic or tolerant phenotype still need to be identified. To obtain a clearer picture of the role B cells and antibodies play, several studies have to be undertaken herein. One major drawback of the studies was the inability to purify drug-specific antibodies. Instead, whole plasma was used as a source of drug-specific antibody. Further studies should include antibody purification as this will enhance assessment of the functional differences between IgG produced from volunteers and naïve, tolerant and hypersensitive patients. This could also facilitate the assessment of differences between non-specific and drug-specific IgG and help to explain the reasons behind the presence of anti-drug antibodies in the absence of hypersensitivity. A cursory search at the downstream signalling pathways and genes up or down regulated by whole drug or drug-modified conjugates in the presence and absence of these antibodies could also be important. In the absence of purified specific antibodies, the generation of B cell lines with the ability to secrete not only IgG but piperacillin-specific antibodies will ensure that a system which closely mimics that obtained *in vivo* and hence mirrors those effects will be in place, limiting the obvious disadvantages associated with the use of whole plasma as a source of our drug-specific antibodies. The characterization of conjugates formed during the course of therapy and their hapten density and molar quantification should also be performed concurrently with that of conjugates generated *in vitro* to ensure clinically relevant scenarios are reproduced in *in vitro* assays. This will help to ensure toxicity in the form of excessive dose is eliminated when conducting experiments.

Nonetheless, I have been able to present relevant data that begins to describe the different molecular events and components of the drug-specific humoral and cellular

immune response that develops in piperacillin hypersensitive patients with CF. The kinetics of drug-specific B and T cell activation may be an important determinant of whether drug exposure leads to an immune response that results in tissue injury.

APPENDIX.

APPENDIX 1

a. Cell lines for B-cell immortalisation

Cell line	Source
B95.8 cells	1. Public Health England, Porton Down, Salisbury.

2 Cell culture drugs, antibodies and substrates for flow cytometry, ELISA, ELISpot and western blot.

Drugs/antibodies	Source
1. Cyclosporin A	Sigma
2. IL-2	Peprotech
3. PHA	Sigma
4. Cpg-dna	
5. ^{3}H thymidine	Moraveck
6. ^{123} [Cs]	
7. CD3, CD4 , CD8 fluorochrome antibodies	BD Biosciences
8. CD19 , CD27 fluorochrome antibodies	BD Biosciences
9. Mouse anti-human penicillin antibody	ABD Serotec
10. Rat anti-flucloxacillin antibody	Gift from Van-pelt.
11. HRP labelled mouse anti-human IgG1 antibody	Invitrogen
12. HRP labelled mouse anti-human IgG1 antibody	Invitrogen
13. HRP labelled mouse anti-human IgG1 antibody	Invitrogen
14. HRP labelled mouse anti-human IgG1 antibody	Invitrogen
15. human serum standard for IgG sub-classes	Nordic
16. BCIP/NBT	Mabtech
17. TMB ELISA substrate	Mabtech
18. Bovine serum albumin (BSA)	Sigma
19. Human serum albumin (HSA)	Sigma
20. Tetanus toxoid	Statens serum institute

APPENDIX 2

a. Cell culture consumables and assay kits.

Consumable	Source
1. IFN-γ, IL-13, IL-5 and granzyme B ELISpot kit/substrates/antibodies	Abcam
2. Human IgG ELISA quantitation test	Bethyl
3. 96U well plates	Nunc 163320
4. 96 flat bottom well plates	Nunc 163320
5. High binding 96 flat bottom well plates	Greiner bio-one
6. 24 well plates	Nunc 142475
7. 48 well plates	Nunc 150687
8. Printed Filtermat A	Perkin Elmer 1450-421
9. Multilex A scintillator sheet	Perkin Elmer 1450-441
10. Sample plastic bag	Perkin Elmer 1450-432
11. 0.2µm filters	Sartorius 16534
12. 0.45µm filters	Sartorius 16534
13. 20 ml tube	Sterilin 128C

APPENDIX 3

b. Chemicals for SDS PAGE western blot and mass spectrometry analysis.

Chemical	Source
1. Tris base	Fisher
2. Skim milk powder	Fluka biochemika
3. Sodium chloride	Normapur Prolabo
4. Ultra-pure protogel	National diagnostics
5. 4 x protogel resolving buffer	National diagnostics
6. N,N,N',N-tetramethylenediamine for electrophoresis (TEMED)	National diagnostics
7. Ammonium persulfate (APS)	Sigma
8. Methanol HPLC grade	Fisher
9. See blue MW marker	Invitrogen
10. Sequencing grade modified trypsin	Promega
11. Plus one Dithiothreitol (DTT)	Life technologies.
12. Iodoacetamide	Sigma
13. C18 Zip tips	

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